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Viral and Bacterial Levels Resulting from the Land Application of Digested Sludge

Metropolitan Sanitary District of Greater Chicago, IL

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Viral and Bacterial Levels Resulting from the Land Application of Digested Sludge

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16. ABSTRACT Surface and ground waters, sludge, soils and aerosols were sampled at a land reclamation site. The site has received large quantities of anaerobically digested sludge for several years. Samples were analyzed for viral and bacterial components to determine the impact of large scale sludge application on the environment. Sixty-eight water samples from streams, reservoirs, wells and runoff were processed for bacteria and viruses. Water samples upstream (S-1) and downstream (S-2) of the site show that the downstream site is lower in total coliform (TC) than the upstream site, while there are no differences in fecal coliform (FC) or fecal streptococcus (FS) levels. Water samples from Reservoir 3 which drains approximately 5,000 acres of land to which sludge has been applied indicate TC levels higher than those in a control reservoir which drains untreated land, with no differences between FC and FS. Six samples contained virus which were confirmed by subpassage. Three of these were found to be contaminated and contained poliovirus 1. Two of the other positive samples were from stream site S-1 and contained echovirus 1 and an unidentified isolate. The other positive sample was from stream site S-2 and contained an unidentified virus isolate. No animal viruses could be confirmed in any well water, sludge or soil samples nor in runoff water from fields to which sludge was applied. LVAS samplers gave total bacterial counts up to cfu/m^3 and coliphage up to 2.2×10^3 pfu/m^3. Andersen sampling gave total viable counts up to 6.6×10^3 cfu/m^3 downwind of the sludge spray apparatus. Upwind values for the LVAS samplers ranged to 5.5×10^2 cfu/m^3 and 1.2×10^2 pfu/m^3 for total bacteria and coliphage respectively. Upwind Andersen samples ranged to 3.6×10^2 cfu/m^3.

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VIRAL AND BACTERIAL LEVELS RESULTING FROM THE LAND APPLICATION OF DIGESTED SLUDGE

by

Metropolitan Sanitary District
of Greater Chicago
Chicago, Illinois 60611

and

IIT Research Institute
Chicago, Illinois 60616

Contract No. 68-02-2223

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HEALTH EFFECTS RESEARCH LABORATORY
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U.S. ENVIRONMENTAL PROTECTION AGENCY
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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The primary mission of the Health Effects Research Laboratory in Cincinnati (HERL) is to provide a sound health effects data base in support of the regulatory activities of the EPA. To this end, HERL conducts a research program to identify, characterize, and quantitate harmful effects of pollutants that may result from exposure to chemical, physical, or biological agents found in the environment. In addition to valuable health information generated by these activities, new research techniques and methods are being developed that contribute to a better understanding of human biochemical and physiological functions, and how these functions are altered by low-level insults.

This report provides an assessment of microbiological levels in surface and ground waters and in aerosols at a land reclamation site receiving large quantities of anaerobically digested sludge, The accumulation of data on microbiological contaminant levels as a result of land application will enable a determination of potential health effects associated with such practices.

R. John Garner
Director
Health Effects Research Laboratory

ABSTRACT

Surface waters, ground waters, sludge, soils and aerosols were intensively sampled at a 15,000 acres (6,070 hectares) Central Illinois land reclamation site during 1975 and 1976. The site has received large quantities of anaerobically digested sludge (~4% solids) for several years (1971-1978). These samples were analyzed for viral and bacterial components to determine the impact of large scale sludge application on the environment. Conventional techniques (MPN and membrane filtration) were used to measure the bacterial parameters. Viruses in water were first concentrated using a modified aluminum hydroxide, continuous-flow centrifugation procedure. The concentrates were assayed for virus using Buffalo Green Monkey (BGM) cells to demonstrate plaque-forming units (pfu) or virus concentration. Viruses were identified by serum neutralization tests. Bacterial viruses (coliphage) were assayed using a combination of MPN or direct plaque assay with Escherichia coli C3000 as host. Soil and sludge samples were assayed for virus by first concentrating the viruses using a polyethylene glycol procedure and subsequent virus assay of the concentrate using BGM cells. Aerosols from a sludge spray application site were captured using either Litton high volume samplers (LVAS) or Andersen six-stage impactors. Fluid from LVAS units was assayed directly for total bacteria, coliphage or animal virus as required.

Sixty-eight (68) water samples from streams, reservoirs, wells and runoff were processed for bacteria and viruses during the fifteen months of this study. Big Creek water samples upstream (S-1) and downstream (S-2) of the site show that the downstream site is lower in total coliform S(TC) than the upstream site, while there are no differences in fecal coliform (FC) or fecal streptococcus (FS) levels. Water samples from Reservoir 3 (R-3) which drains approximately 5,000 acres (2,023 hectare) of land to which sludge has been applied indicate TC levels higher than those in R-10, a control reservoir which drains untreated land, with no differences between FC and FS. Of the 68 water sample concentrates, six contained virus which were confirmed by subpassage. Three of these were found to be contaminated and contained poliovirus 1, sabin strain. Two of the other positive samples were from stream site S-1 and contained echovirus 1 and an unidentified isolate. The other positive sample was from stream site S-2 and contained an unidentified virus isolate. No viruses could be confirmed in any well water

samples. Bacteriological data from the well water samples was of an indeterminate nature.

No animal virus could be detected in any sludge or soil samples nor could virus be detected in the sludge spray source used for surface application. No virus were confirmed in runoff water from fields to which sludge was applied.

Aerosol studies performed during three separate sampling periods were difficult to assess. Statistical analysis of the data indicated that LVAS samplers gave total bacterial counts from 10^4-10^6 colony forming units (cfu)/m^3 and for coliphage from 0-2.2×10^3 pfu/m^3. The Andersen six-stage sampler gave total viable counts in the range of 5.8×10^1 - 6.6×10^3 cfu/m^3, downwind of the sludge spray apparatus, upwind values for the LVAS samplers ranged from 1.5×10^2 - 5.5×10^2 cfu/m^3 and 0-1.2×10^2 pfu/m^3 for total bacteria and coliphage respectively. Upwind values taken with Andersen six-stage samplers ranged from 4.6×10^1 - 3.6×10^2 cfu/m^3.

Four (4) ambient air samples (1 upwind, 3 downwind) had detectable confirmed virus levels. Confirmations were based on second or third blind passage in homologous cells. Aerosol data were subjected to rigorous statistical analysis.

Laboratory studies were conducted during the course of these investigations to determine the efficiency of the two virus concentration techniques and to simulate virus travel in the Fulton County soils. The $Al(OH)_3$ continuous-flow centrifugation technique had a recovery efficiency for seeded water samples of 67% using Hep 2 cell cultures to 333% in BGM cell cultures, with poliovirus type 1 as test organism. The polyethylene glycol the hydroextraction procedure recovery efficiency ranged from 13.7 - 44.4% using BGM cell cultures and poliovirus type 1. Poliovirus type 1 and echovirus 7 were shown to adsorb very readily to Fulton County soil; with penetration through a saturated soil limited to the top 1 or 2 cm. Assuming identical recovery efficiencies of seeded viruses in soil columns at the beginning and end of these experiments one can estimate virus inactivation of 85 and 82% for poliovirus 1 and echovirus 7 respectively.

This report was submitted in fulfillment of contract No. 68-02-2223 by the U.S. Environmental Protection Agency.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

A6S	-- Anderson six-stage air sampler
BGM	-- Buffalo Green Monkey
cfs	-- cubic feet per second
cfu	-- colony forming units
cpe	-- cytopathic effect
EDTA	-- disodium ethylenediamine tetraacetic acid
E-MEM	-- minimum essential medium with Earle's salts
FCS	-- fetal calf serum
g	-- gravity
HBSS	-- Hank's balanced salt solution
HIFCS	-- heat-inactivated fetal calf serum
iu	-- international UNF
LVAS-M	-- large volume air sampler, Litton-model M
MPN	-- most probable number
mpnpfu	-- most probable number of plaque forming units
MSDGC	-- Metropolitan Sanitary District of Greater Chicago
PAB	-- phage assay broth
PABA	-- phage assay broth agar
PBS	-- phosphate buffered saline
pfu	-- plaque forming unit
$TCID_{50}$	-- 50% end-point tissue culture infectious dose
TSA	-- trypticase soy agar
v/v	-- volume per volume
w/v	-- weight per volume

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SECTION 1

INTRODUCTION

The use of anaerobically digested municipal sludge as a fertilizer and soil amendment is becoming a widely accepted method of sludge disposal both in Europe and the United States (1-3). By this practice, plant nutrients are recycled, and an economical non-polluting sludge disposal method can result.

The Metropolitan Sanitary District of Greater Chicago (MSDGC) has implemented a program whereby processed sludge is used for the reclamation of strip mined fields and fertilization of row crops in Fulton County, Illinois. Waste activated sludge and some primary sludge are thickened, anaerobically digested, shipped by barge 200 miles down the Illinois Waterways and finally lagooned before land application. The product of this treatment is an organically stabilized liquid fertilizer (4).

Despite the extensive processing, the possibility of increased virus and bacteria in the environment resulting from sludge application is of concern to the MSDGC and other agencies. For this reason, the various processes employed in the production of liquid fertilizer are being examined in an effort to model the fate of viruses and bacteria initially present in sewage. In addition, an extensive monitoring program is being established to determine the possible presence of sludge-associated bacteria and viruses in the environment as a result of the land application of this liquid fertilizer.

Enteric bacteria and viruses are present in sewage. The activated sludge sewage treatment process results in the adsorption of bacteria and viruses to the activated sludge floc (5). A portion of this floc, the waste activated sludge, is later anaerobically digested. Enteric bacteria and viruses associated with the waste activated sludge would, therefore, be carried into the anaerobic digestion unit process. Following an average digestion period of 14 days and shipment to Fulton County, the processed material is lagooned for at least 60 days before being applied to strip mined fields and placed on land by spray irrigation of subsurface injection into the soil with disc equipment. For spray irrigation, water-winch brand sprayers were used. Orifices of 1-1/2" to 2" and up to 90 psi sludge pressure were used. Each field

which receives sludge is completely bermed and drained solely into a runoff capture basin which was designed to hold at least the 100 year storm. Each basin had a control structure so that only regulated release of runoff water into receiving waters was possible (Figure 1).

The objective of this study was the assessment of the effects of the land application of digested lagooned sludge upon the viral and bacterial content of contingent surface waters, ground water, soil and air at the Fulton County land reclamation site. The Fulton County site is representative of land reclamation programs (1-3) in areas of similar soil and environmental characteristics.

The overall study consisted of two components. The first component was concerned with the development of methodologies for the concentration and isolation of viruses from water, sludges and soils. Upon establishment of the appropriate methodologies, the various elements of the second component were initiated.

The second component of the study included the monitoring of various environments at the land reclamation site for viruses and indicator bacteria. The environments monitored included 3 surface water sites, 3 ground water sites, a site associated with a field runoff basin, lagooned sludge, a field where lagooned sludge was injected into the soil and 4 fields where aerosols were generated during the spray application of lagooned sludge.

In addition, a laboratory study was conducted to assess the movement of two types of viruses seeded onto soil columns in the laboratory. The soils used in this study were taken from the land reclamation site.

The specific work items established for the entire program were:

1. Surface Water Monitoring - Two stream sites (S-1 and S-2) and one reservoir site (R-3) (see Figure 2) were monitored for viruses and indicator organisms monthly during the study. The surface water monitoring was to estimate the transfer of viruses possibly contained in sludge to surface drainage. To this end, also, an MSDGC land application area (Field 13) was selected and closely monitored for viruses. Sludge applied to fields, runoff water from rain events, and release water and sediments from the field runoff capture basin (B-13) were examined for viruses. In addition, the stream into which the runoff capture basin discharged was examined both upstream and downstream of the basin discharge (see Figure 2).

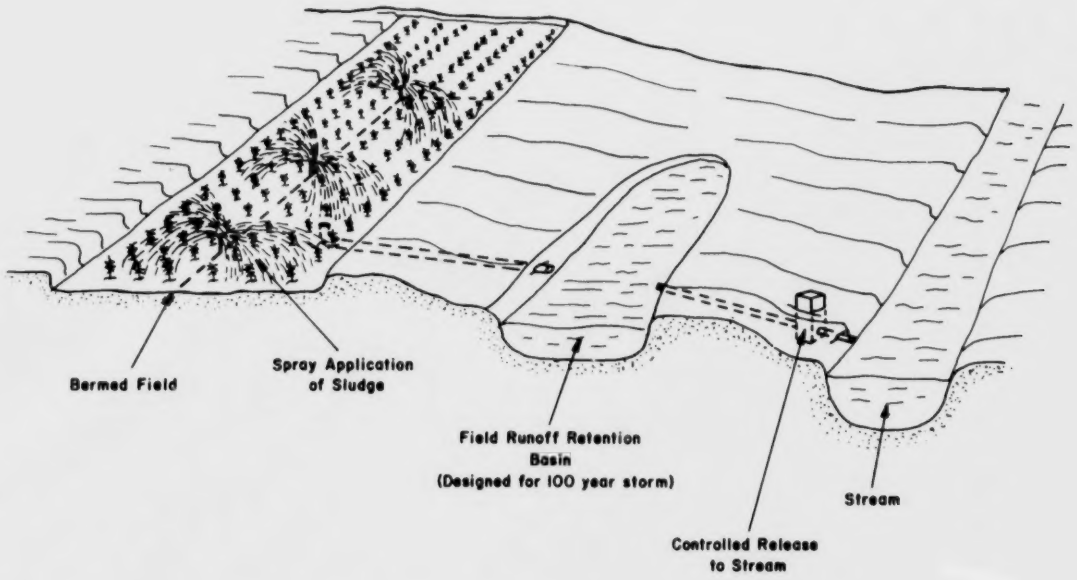
Bermed Field
Spray Application of Sludge
Field Runoff Retention Basin (Designed for 100 year storm)
Controlled Release to Stream
Stream

Figure 1. Typical field design with runoff water capture system
Fulton County, Illinois

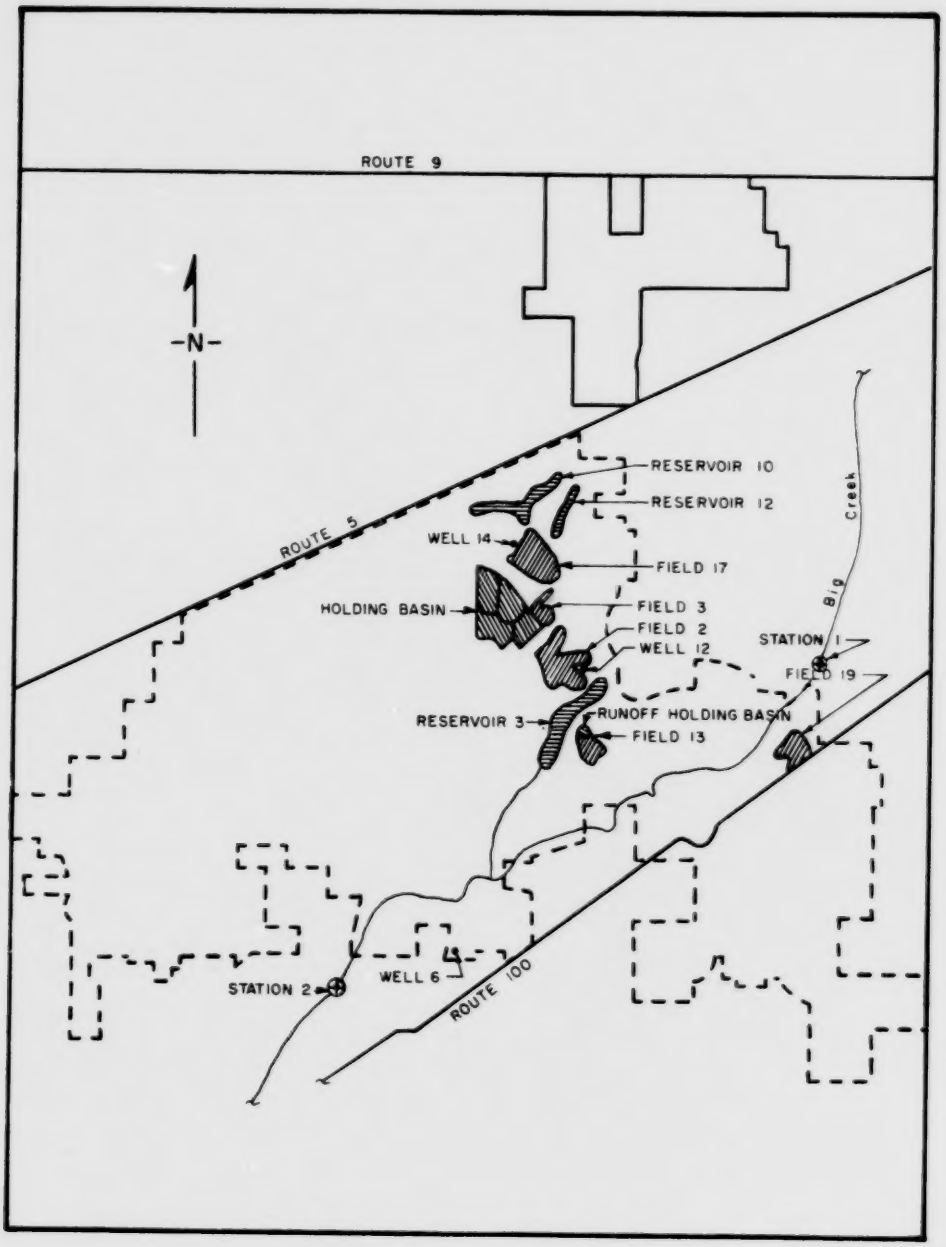

Figure 2. Map Fulton County Land Reclamation Site

2. Methodology Development and Verification - Procedures for concentrating and isolating viruses from water, soil and sludge were developed and verified.

3. Determination of Virus Survival in Sludge Lagoons - Virus levels, in fresh digested sludge, were monitored over a 2 month period to determine the die-away rate. These studies were carried out utilizing a small pilot lagoon constructed and filled with fresh digested sludge for this purpose.

4. Virus Movement in Soil - Laboratory study - Soil columns were set up with Fulton County soil. Sludge seeded with known amounts of 2 human viruses were applied to these columns, and rainfall was simulated by addition of water to the columns daily for a period of 5 days. Virus in the leachate and at various depths within the column were determined.

5. Virus Movement in Soil - Groundwater study - Wells in the vicinity of the sludge holding lagoons at the MSDGC Fulton County site (see Figure 2) were monitored to assess virus transport from the sludge to the groundwater.

6. Aerosol Studies - During application of lagooned sludge to Fields 2, 3, 17, and 19 (see Figure 2), aerosols were captured and the animal virus, coliphage and bacterial levels were determined. Large volume air samplers as well as Anderson six-stage samplers were employed for these determinations. Sludge source samples were also assayed for virus and bacteria.

SECTION 2

SUMMARY

1. After digestion and storage in open lagoons, no viruses were detected in sludge used at the Fulton County site.

2. Bacterial and viral analysis of surface water and runoff water indicates that the land application of sludge did not affect the quality of these waters.

3. Well water analysis indicate no effect of sludge application upon the microbiological quality or groundwater at the Fulton County site.

4. Statistical analyses of aerosol data indicate that prior conditions influence downwind recovery of animal virus.

5. Only four (4) of the twenty-two (22) aerosol samples showed detectable animal viruses, no viruses were detected in the sludge source, and all detected viruses were of the same serotype-poliovirus 1. The possibility of contamination of these samples could not be ruled out.

6. Coliphage levels decreased exponentially as the square root of the distance of the sampler from the spray source.

7. Bacterial counts in aerosols appeared to be directly influenced by the wind velocity and inversely affected by distance and temperature, when estimates are made using Andersen six-stage samplers.

8. Bacterial counts in aerosols appeared to be directly influenced by temperature and distance, when estimates are made using Litton high-volume samplers.

9. Movement of seeded poliovirus 1 and echovirus 7 was limited to the top 1.3 cm of soil columns in laboratory experiments.

10. Relative humidity did not seem to affect estimates of bacterial aerosols using either sampler.

SECTION 3

MATERIALS AND METHODS

CELL CULTURES AND MEDIA

The continuous African green monkey kidney cell line designated Buffalo Green Monkey (BGM) was obtained in its 136th passage from Dr. Flannagan of the State University of New York at Buffalo or International Biological Laboratories (Rockville, Md.). Embryonal rhabdomyosarcoma; Human, cell cultures (RD) were obtained from the American Type Culture Collection (Bethesda, Md.). Epidermoid carcinoma, larynx; Human, cells (HEP 2) were obtained from the Illinois Department of Public Health.

RD cell cultures were passaged on growth medium consisting of minimum essential medium with Earle's salts (E-MEM) containing 10% (v/v) fetal calf serum (FCS) and 50 ug/ml of gentamicin and 2.5 ug/ml amphotericin B. BGM cell cultures were passaged on the same medium except on alternate passages 100 ug/ml chlorotetracycline was added to suppress possible mycoplasma contamination. Monolayer cultures were maintained with E-MEM supplemented with 2% (v/v) heat inactivated fetal calf serum (HIFCS) and 200 iu/ml penicillin G, 200 ug/ml streptomycin sulfate and 2.5 ug/ml of amphotericin B. Plaque assay medium consisted of E-MEM supplemented with 2% (v/v) HIFCS, nonessential amino acids, 0.0017% (w/v) neutral red, 50 ug/ml gentamicin and 5 ug/ml amphotericin B and 1.5% (w/v) Noble agar or purified agar (Difco Laboratories, Detroit, Mich.).

Stock cultures were maintained in Bellco roller bottles with a surface area of 1100 cm^2 at a speed of about 2 R.P.M. at 35° - 37°C and were passaged weekly with a split ratio of 1:4. To passage the cell cultures, confluent monolayers of cells growing in roller bottles were washed 3 times with Hank's balanced salt solution without calcium and magnesium, (HBSS w/o Ca^{+2} + Mg^{+2}). Following this, HBSS w/o Ca^{+2} + Mg^{+2}, containing 0.25% (w/v) trypsin and 0.02% (w/v) disodium ethylenediamine tetraacetic acid (EDTA), was added to the cultures and the cultures were rotated at about 2 R.P.M. until the cells sloughed off the flask. The cell contents of one roller bottle were then suspended in 1 liter of the growth medium and 250 ml of the suspension were added to each of 4 roller bottles to continue the seed cells. The roller

bottles were prepared by gassing them in a 5% (v/v) CO_2 incubator immediately following washing and sterilizing.

For the production of petri dish cultures and microtiter cultures for virus assay, the cell contents of a roller bottle were suspended in 1 liter of the growth medium except that the antibiotic constituents were 250 iu/ml penicillin G and 250 ug/ml streptomycin sulfate. This cell suspension was dispensed in 12 ml amounts into 100 mm diameter plastic petri dishes or in 0.2 ml amounts into the wells of disposable flat bottom microtiter tissue culture plates (Cook Engineering). The petri dish and microtiter cultures were incubated in a humidified incubator under 5% (v/v) CO_2. The cultures generally became confluent within 3 to 5 days at which time they were used for virus assays.

LABORATORY VIRUS STOCKS

Poliovirus 1, Sabin strain, (Abbott Laboratories, North Chicago, Illinois) and coxsackievirus B4 (University of Illinois Medical School, Chicago, Ill.) were propagated in BGM cell cultures. Harvested materials were stored in 1.5 ml aliquots at -70°C. The infectivity titers of the viruses were $10^{8.5}$ and $10^{5.6}$ 50% endpoint tissue culture infectious doses ($TCID_{50}$)/ml respectively. Virus preparations were passed through 0.22 um Millipore or Nuclepore filters prior to use. Millipore filters were pretreated with 1 ml of HIFCS to minimize virus adsorption. Nuclepore filters required no pretreatment.

INFECTIVITY TITRATIONS

Stock viruses and virus isolates were serially diluted 10-fold in maintenance medium. A 0.1 ml amount of each dilution was inoculated in quadruplicate into microtiter plates containing monolayered BGM cells. The cultures were incubated at 37°C in a 5% (v/v) CO_2 atmosphere and were observed daily for one week for cytopathic effect (CPE). Infectivity titers were determined by the method of Reed and Muench (6).

PLAQUE ASSAY TECHNIQUE

Monolayered BGM cells in 100 mm tissue culture dishes (Corning) were inoculated with 0.1 to 1 ml of the appropriate sample or dilution of a sample and were allowed to stand for 2 hours at room temperature for virus adsorption. For the assay of viruses contained in environmental samples, plates were washed twice with Hank's balanced salt solution (HBSS) containing 2.5 ug/ml amphotericin B, 200 iu/ml penicillin G and 200 ug/ml streptomycin sulfate. Cultures were then overlayed with 15 ml of plaque assay medium. The plates were incubated at 37° under 5%

(v/v) CO_2 for at least 8 days and were observed daily for plaque formation. As a control, an appropriate dilution of poliovirus 1 (50-100 plaque forming units (pfu)/ml) was plaqued in each experiment. The test was assumed valid when 50-100 plaques were observed on the control plates. Sample toxicity controls consisting of 2 ml aliquots of each concentrate mixed with 1 ml of control poliovirus were also included in each assay. Positive plaques were subpassaged at least once in BGM cells before recording a sample as positive or negative for virus.

IDENTIFICATION OF VIRUS ISOLATES

Serum neutralization tests were performed in Falcon multiwell dishes (Falcon Plastics) containing 24 wells. The dishes contained 24-hour cultures of BGM or RD cells. Twenty-five antibody units in 0.025 ml of Lim Benyesh-Melnick horse antiserum pools A through H (National Institute of Allergy and Infectious Disease) were mixed with 100-320 $TCID_{50}$ per 0.025 ml of viral isolate and incubated at 37°C for 2 hours. A 0.05 ml amount of each virus-serum mixture was added in duplicate to wells containing 1 ml of E-MEM with 0.5% (v/v) FCS. Plates were incubated at 37°C in 5% (v/v) CO_2 and observed daily for CPE. Specific neutralization tests were conducted by the same methods using horse antisera to echovirus 9, Hill; exhovirus 17, CHHE; echovirus 21, Farine; and poliovirus 3, Leon; and Rhesus monkey antisera to poliovirus 1, Brunhilde; and poliovirus 2, Lansing.

Physical Characterization

Ether sensitivity was determined by exposing a 1:10 dilution of a virus isolate to 20% ethyl ether for 18 hours at 40°C.(7)

Acid resistance was determined by making a 1:10 dilution of the virus isolate in HBSS, then lowering the pH to 3.0 for 3 hours at room temperature.(8)

Temperature markers were differentiated by inoculating confirmed poliovirus isolates into BGM cell cultures and incubating at 37°C and 40°C to differentiate virulent and attenuated strains. (9)

MYCOPLASMA DETECTION

Concentrates of cell cultures and selected virus isolates were tested for presence of mycoplasma.(10) One milliliter of each sample was inoculated into 10 ml of Difco PPLO (pleuropneumonia-like organism) broth without crystal violet, supplemented with Difco Mycoplasma Supplements. A 0.1 ml aliquot of the test sample was also inoculated onto duplicate petri dishes containing

mycoplasma broth with 0.9% (w/v) Oxide Ionagar No. 2. One plate was incubated aerobically and the other anaerobically at 37°C. After 4 days of incubation, broth cultures were transferred to fresh petri dishes and broth cultures and incubated. These broth cultures were transferred to agar plates after an additional 4 days of incubation. All plates were examined microscopically (300x) at 2 to 3 day intervals for 14 days before being discarded as negative. Presumptive Mycoplasma would be confirmed by Dienes stain retention, demonstration of subsurface colony growth, and ability to grow when subcultured. Mycoplasma were not detected during the study.

CONCENTRATION OF VIRUSES FROM WATER SAMPLES

For the water samples collected August 18, 1975 and September 23, 1975, 2 virus concentration techniques were used. Viruses from 4 liter creek samples were concentrated approximately 1000 fold by means of the aluminum hydroxide ($Al(OH)_3$) procedure (11). Solids removed from the prefiltration filter by means of a spatula were suspended in 15 to 25 ml of a medium containing 0.05M glycine at pH9 and 3% (w/v) disodium EDTA. The mixture was sonified in a sonic water bath (Cole-Parmer, Model 8845-6) for 20 minutes. For sonication the material was placed in 16mm by 150mm screw cap tubes and suspended in the sonic water bath by means of a wire. The above preparation was then centrifuged at approximately 3000 x g for 30 minutes prior to filter sterilization of the supernatant employing a serum treated 25 mm Millipore type HA (0.45 um) filter. For treatment of the above filter, 20 ml of 10% HIFCS contained in phosphate buffered saline (PBS) were passed through the filter with a syringe. Sterilized filtrates were maintained at -70°C until virus assay. Viruses from 20 liter reservoir samples were concentrated by the PE 60 method (12). Solids obtained by prefiltration were treated as described above for the $Al(OH)_3$ procedure.

After September 23, 1975, viruses from all water samples were concentrated using an $Al(OH)_3$- continuous flow centrifugation technique. A Sorval Model RC 2B centrifuge and Sorval KSB continuous flow system were used. All metal apparatus that came in contact with the sample was autoclaved or disinfected with Roccal. Rubber tubing was autoclaved or replaced. Samples were adjusted to pH 6.0 and 10 ml of freshly prepared $Al(OH)_3$ were added per liter of water sample. After a 2-hour mixing period in an ice bath during which pH was held constant, the sample was centrifuged at 27,000 x g at a flow rate of 200 ml/min. The sediment was resuspended in pH 10.5 glycine buffer (0.05M) containing 2% (w/v) disodium-EDTA and 10% (v/v) HIFCS and adjusted to pH 9.0 with 0.5M NaOH while stirring constantly for 10 minutes. The concentrate was sonicated (Branson sonifier Model S 125) for 10 to 20 sec. at 4.5 amps, mixed for 20 min to elute the virus and centri-

fuged at 27,000 x g (Sorval RC 2B with SS 34 rotor) for 15 min. to remove particulates. The supernatant was collected and filtered through a HIFCS treated, 25 mm, 0.45 um Millipore or 0.2 um Nucleopore membrane filter. The filtrate was mixed with an equal volume of HBSS containing 3% (w/v) beef extract, 1% (w/v) gelatin and 5% (w/v) $MgSO_4$ to stabilize the virus.(13) Aliquots were frozen at -70°C until assayed.

CONCENTRATION OF VIRUSES FROM SLUDGE AND SLUDGE-SOIL SAMPLES

Viruses in sludge and sludge-soil samples were concentrated by a modification of a Polyethylene glycol hydroextraction method. (14) A 200 ml volume of sludge or a 200 gram quantity of sludge-soil sample was diluted to twice its volume by adding glycine buffer to a final concentration of 0.05 M. Tween 80 was added to give a concentration of 0.5% (v/v) and the mixture was adjusted to pH 10.5 with 1M NaOH. The mixture was stirred for 15-20 minutes on a magnetic stirrer at 4°C and then sonified for 3 minutes with a Branson W 350 sonifier. The power setting (output control) was 7 and percent duty cycle setting was 60%. The standard probe was used. The sonified mixture was centrifuged at 9400 x g at 4°C for 30 minutes in a Sorval RC 5 refrigerated centrifuge using an HS-4 rotor. The supernatant was adjusted to pH 7.5 with 2N HCL and twice treated with Freon 113 (Dupont). For Freon treatment, an equal volume of Freon 113 was added to the supernatant and the mixture was stirred for 30 minutes at 4°C. The aqueous layer was removed with a pipette, without disturbing the interphase, and centrifuged at 9.400 x g for 60 minutes at 4°C. Gentamicin and amphotericin B were added to the supernatant to give final concentrations of 62.5 ug/ml and 6.25 ug/ml respectively and the supernatant was dialyzed for 18-24 hours at 4°C against polyethylene glycol (Carbowax 6000, McKesson Chemical). The concentrate was collected in a sterile tube. The inner surface of the dialysis bag was twice washed with 15-25 ml of E-MEM containing 2% (v/v) HIFCS. The washings were pooled with the concentrate. The concentrated mixture was sonified for 30 seconds at a power setting (output control) of 7 and the percent duty cycle set at 60%. A microtip probe was used. During sonication the tube containing the sample was held in an ice-water mixture. The sonified mixture was centrifuged in a Sorval RC 5 with a SS-34 rotor at 40,000 x g for 60 minutes at 4°C. The supernatant was maintained at -20°C until assayed for viruses.

CONCENTRATION OF MICROORGANISMS FROM AIR

Air samples for animal virus and coliphage assay were collected by methods that were essentially those described by Fannin, et al.(15) Bacterial samples were collected by these methods when (a) Andersen samplers were not available to the project and

(b) when the sludge spray apparatus was shut down prior to Andersen sample collection but after the commencement of large-volume air sample. The Large Volume Air Samplers, Model-M (LVAS-M), (Litton Systems, Inc.) were operated at an air sampling rate of 1 m^3/min. with 14.0-15.0 kilovolts through the electrostatic precipitators and a sampling fluid flow rate of 7 to 9 ml/min. depending upon environmental conditions. These samplers contained qutomatic devices to recirculate the sampling fluid through the LVAS-M while replacing water lost through evaporation. A sampling fluid, consisting of 30 ml of PBS, containing 2% (v/v) of a 1% (w/v) phenol red solution, 0.03% (v/v) GE Antifoam 10, and 2% (v/v) HIPCS was used. The reservoir containing this fluid was kept in an ice bath during the sampling period.

After sampling, the fluid was collected in sterile Vacutainer tubes and kept on ice until frozen at the end of each sampling day. After each field sampling period, the samples were stored at -70°C until assayed.

Between samples, the following disinfection protocol was used: LVAS tubing was washed with at least 400 ml of a greater than 25% 7X detergent solution, 1000 ml sterile distilled water, and 500 ml sterile triple distilled water.

Nontubing attachments to each sampler were autoclaved (15 lbs., 15 min.) prior to each use. When collection of total bacteria with the LVAS was intended, 0.1 ml of final wash water was plated on trypticase (TSA) by the spread plate procedure. No growth was observed on these plates.

Processing of Samples

Each LVAS-M air sample, contained in sampling fluid, was thawed and the volume measured. After thorough mixing of the entire sample, the initial pH was measured and recorded. The pH was then adjusted to 9.0 with 0.1 N NaOH and the sample was sonicated for 15 sec. with a Branson S 125 Sonifier at 4.5 amps using the standard probe.

Following centrifugation at 940 x g for 30 minutes, the sample was filtered through a 0.45 um Millipore filter pretreated with HIFCS. The pH was then adjusted to 7.0 with 0.1 N HCl and the sample was placed in 2.5 ml aliquots in sterile Vacutainer tubes which were frozen at -70°C until assayed.

Portions of liquid sludge source samples for coliphage assay were processed by initial Vortex mixing followed by sonication as described above. The sample was then centrifuged at 940 x g for 30 minutes and the supernatant filtered through a 0.45 um Millipore filter pretreated with HIFCS.

Sludge source samples were processed for animal virus isolation employing the procedure described in the previous section.

Sample Assay

Samples were plaque-assayed for animal viruses in BGM cells as described. Prior to air sample inoculation, monolayers of BGM cells were washed twice with HBSS. Aliquots taken in experiments to evaluate virus concentration and isolation techniques were assayed in BGM or Hep 2 cell culture.

Coliphage and total bacterial assays were run using the following protocol. Cultures of Escherichia coli C3000 grown for 4 hours in phage assay broth (PAB) were used for coliphage assays. PAB was prepared by adding distilled water to 8.0 gm nutrient broth; 5.0 gm NaCl; 0.20 gm $MgSO_4 \cdot 7 \cdot H_2O$; 0.05 gm $MnSO_4 \cdot H_2O$ to a final volume of 1 liter. After dissolving these ingredients, 0.15 gm $CaCl_2$ was added. The phage assay broth agar (PABA) was prepared by adding 7 or 15 gm Difco agar to 1000 ml of PAB. Soft agar (0.7%) was used for preparation of a seeded host lawn or for coliphage plaquing over a hard agar (1.5%) base.

The procedure described by Chang, et al (16) was used for most probable number plaque forming unit (mpnpfu) calculations. Coliphage assays on air sample concentrates were made by the most probable number (MPN) procedure as described by Fannin, et al (14) or, when endpoints were not reached with this method, by plaquing 10-fold dilutions by the soft agar overlay method. (17)

For the MPN procedure, fourfold dilutions of each sample were inoculated into five replicate tubes of 10 ml of PAB. To each of the inoculated tubes, 0.1 ml of a 4-hour culture of E. coli C3000 was added. Following Vortex mixing, the tubes were incubated overnight. Each tube was assayed for phage growth by spotting with a sterile wooden applicator stick a drop from each tube onto a freshly seeded lawn of a 4-hour E. coli C3000 - soft PABA (0.7% agar) suspension on top of an agar base. The soft agar overlay was performed by inoculating 2.5 ml of melted soft PABA at 45°C with 0.1 ml of a 4-hour culture of E. coli C3000. One ml of the assay dilution was inoculated into this host-PABA suspension, mixed on a Vortex, and poured onto a prepared hard PABA base plate. The mixture was gently tilted to cover the entire agar plate surface. All plates were incubated for 5 to 8 hrs. and observed for lysis or plaque formation. Positive and negative phage controls were included with all tests.

Total bacteria from LVAS-M sampling fluid were assayed on

TSA in duplicate by the spread plate method and incubated for about 24 hours at 37°C. Plates taken with Anderson six-stage air sampler (A6S) were incubated under similar conditions. Results were reported as colony forming units (cfu).

ENUMERATION OF BACTERIA

The indicator bacteria: Fecal coliforms, total coliforms and fecal streptococci in surface and well water samples were enumerated according to Standard Methods.(18)

STATISTICAL ANALYSES

Statistical methods and analyses of bacteriological data are presented in Appendix A, and of virological data in Appendix B.

SECTION 4

EXPERIMENTAL PROCEDURES

WATER SAMPLES

Surface and ground water samples were collected and processed for virus isolation and identification, and enumeration of indicator bacteria. The stations S-1 and S-2 were respectively the points where Big Creek enters and leaves the MSDGC land reclamation property. Station R-3 is at the outlet of Evelyn Reservoir which drains a large portion of the sludge treated area (Figure 2).

Before July, 1976, samples from Big Creek (S-1 and S-2) were obtained by means of automatic samplers (Pro Tech, Model CG 125) and were 24-hour composites. Samples from Evelyn Reservoir (R-3) were grab samples. Because no viruses were detected, sample volumes for S-1 and S-2 were increased from 4 liters to about 20 liters after the June, 1976 sample. Because the automatic composite samplers could not collect 20 liters of sample, the grab method was used. Approximate four liter grab samples were collected from the outfall of basin B-13-1 (B-13-1c), above the discharge (B-13-1a) and 10 feet downstream of the basin discharge (B-13-1b). Runoff basin B-13-1 discharges into Evelyn Creek which drains R-3. Twenty to 40 liter water samples were collected from Well 6, Well 12 and Well 14 (Figure 2). The wells were sampled in order to assess the movement of viruses from the sludge lagoon to the ground water. Wells 12 and 14 are in very close proximity to over 260 acres of sludge holding basins some of which are over 50 feet deep. In the original work plan, Well 12 was to be monitored on a bimonthly basis. The pump in the well was malfunctioning at the beginning of the study, however, and Well 14 was monitored instead. Well 6 was a control well far removed from the sludge application and sludge holding basin areas.

Direct field runoff was collected from Field 13 (RT-13), by burying a two-inch deep trough in the upper soil in the field. Two rainfall events occurred during the course of the study for which water samples were collected and processed.

Water samples were collected, packed in wet ice, and shipped to Chicago. They were received within 24 hours. The samples

were processed immediately upon receipt or after 24 hours of storage at 4°C.

Virus assay of these concentrated water samples was performed using the plaque technique. The total number of virus plaques was recorded.

Virus plaques, selected on the basis of size and morphology, were picked, resuspended in maintenance medium and frozen at -70°C for subpassage. A minimum of 10 plaques were picked from plates inoculated with a concentrate. All plaques were picked if less than 10 were observed.

When CPE was observed, cells and supernatant fluids were harvested and frozen for identification. After infectivity titrations, attempts were made to identify the agents by serum neutralization tests or by physical characterization.

Samples were processed by methods developed in the MSDGC laboratory in conjunction with Illinois Institute of Technology Research Institute (IITRI). To evaluate the efficiency of the $Al(OH)_3$-continuous flow centrifugation technique, four typical ground and surface waters were inoculated with known concentrations of stock poliovirus 1 and coxsackievirus B4. Concentrates were assayed for virus using the plaque assay or infectivity titrations.

SLUDGE AND SLUDGE-SOIL SAMPLES

In this phase of the work, the existence of viruses in anaerobically digested sludge and sludge-soil environments was assessed.

Pilot Sludge Holding Basin

Virus survival, with time, was examined in a field pilot sludge holding basin (41m x 27m x 6m). The pilot holding basin was filled with sludge piped 10.4 miles from barges in Liverpool, Illinois on the Illinois River. Samples were obtained from the lagoon as it was filled and at weekly intervals thereafter. After the sludge in the basin separated into a supernatant and sediment fraction (approximately 1 week after filling the basin) both fractions were sampled simultaneously. Twelve samples were examined for virus content.

Field 17

Sludge-soil samples were collected at approximately weekly

intervals from Field 17 (Figure 2) after sludge was incorporated into its topsoil. Three samples were examined for virus content.

Runoff Basin

Because of infrequent rainfall, the runoff basin (B-13) was discharged only once during the study. Therefore, to supplement the runoff basin information, the sediment on the bottom of runoff basin 13-1 (B-13-1) was sampled periodically and processed for viruses. Four samples were examined for virus content during June, September, and October.

VIRUS MOVEMENT IN SOIL - LABORATORY STUDY

The purpose of this study was to estimate the extent of virus penetration into water-saturated columns of Fulton County soil. Table 1 lists some characteristics of the soil used. Poliovirus 1, and echovirus 7, suspended in sludge or distilled water were each applied to the surfaces of columns containing soil. The columns (12.1 cm in diameter by 30.5 cm in length) were made of plexiglass and contained 3.5 kg of soil each. The soil was sieved to exclude particles greater than 2 mm in diameter. Prior to the application of viruses in water or sludge, the columns were saturated with distilled water. The columns were prepared by adding 100 ml of distilled water per day to the tops of the columns and recording the eluate volumes. After one to two weeks, the daily eluate volumes stabilized at approximately 75 to 95 ml per day. The balance was assumed lost by evaporation. For poliovirus 1, 100 ml of digested sludge seeded with 4.3×10^7 pfu were applied to three columns, 100 ml of unseeded digested sludge were applied to a fourth column and 100 ml of distilled water seeded with 4.3×10^7 pfu were applied to a fifth column. For echovirus 7, 100 ml of digested sludge seeded with 2.6×10^8 pfu were applied to four columns, 100 ml of unseeded digested sludge were applied to a fifth column and 100 ml of distilled water seeded with 2.6×10^8 pfu were applied to a sixth column.

Immediately after adding the sludge and distilled water for each virus, the contents of one of the columns containing sludge and seeded virus was completely mixed and an unmeasured aliquot of approximately 2.0 gm was taken, suspended in 2 ml of distilled water and volumetrically equally split for solids determination and virus assay. For virus estimation, the split aliquot was suspended in 4 ml of HBSS containing 3 % (w/v) beef extract, 1% (w/v) gelatin, 2.5 ug/ml amphotericin B and 200 ug/ml gentamicin. Distilled water was added to each remaining column at a rate of 100 ml/day for 5 days. This is equivalent to rainfall of 1.70 inches.

TABLE 1. SOME CHARACTERISTICS OF THE FULTON COUNTY SOIL USED IN SOIL COLUMNS(18)

Parameter	Soil
pH	7.4
Organic carbon, %	0.61
Electrical Conductivity	1.29
Cation Exchange Capacity	14.3
NH_4-N, ug/g	6.59
NO_2+NO_3-N, ug/g	1.77
0.1N HCl extractable	
Zn, ug/g	31.7
Cd, ug/g	0.20
Cu, ug/g	4.79
Ni, ug/g	6.7
Texture	loam, silty clay loam, clay loam
Bulk density, g/cc	1.61

In order to estimate possible virus penetration through the entire length of the columns, daily for 5 days, 1 ml of eluate from all of the remaining columns was collected for virus assay. The eluate samples were diluted in 4 ml of HBSS containing 3% (w/v) beef extract: 1% (w/v) gelatin, 2.5 ug/ml amphotericin B and 200 ug/ml gentamicin.

After 5 days, for each virus, a column containing seeded sludge was thoroughly mixed and sampled as described above for the first columns. A comparison of the virus content of these mixed columns with the virus content of the above described columns, sampled five days previously gave an estimate of virus inactivation in the columns during the study.

For each virus, a seeded sludge column was frozen solid and the plexiglass casing was broken off. Aliquots of soil were removed with a 3/8" cork borer at the circumference of the columns at approximate depths of 1.3 cm, 2.6 cm, 5.1 cm, 10.2 cm, 20.4 cm and the bottom of the columns. The aliquots removed were treated as described for the soil samples taken from the mixed columns at the beginning and the end of the experiments.

AEROSOL SAMPLES

Samples for total bacteria assay were taken with the LVAS-M during the May 24 sampling period. After A6S samplers became available for the project, they were used in all subsequent tests (excluding 3 samples during the August 30 sampling period where the sludge sprayers shut down prior to A6S aerosol collection). Each sampler was loaded with 6 plates, each containing 27 ml of TSA. The A6S were selected for use in order to obtain an aerodynamic size distribution of total bacteria-containing particles at the location of LVAS-M sampling. The total bacterial counts indicate the relative concentrations of microbial contamination arising from direct spraying of sludge as well as other nonspecific sludge application activities. During each sample collection period, wind direction was observed and velocity was measured with a hand-held anemometer. Relative humidity was measured with a battery-operated psychrometer. Air temperature, sky condition, precipitation, and daylight or darkness were also noted. Atmospheric stability classes were estimated by the method referenced by Lighthart and Frisch.(20) Efforts were made to take upwind samples following downwind sampling. Due to periodic shifts in wind direction, these samples were occasionally subjected to downwind influences from the spray source. Sludge source samples for assay of animal virus, coliphage, and total bacteria were taken from an outlet at the sludge pump station near the end of each sampling day.

The source sample was assayed for total bacteria within 2 hours after collection. All samples for coliphage and animal virus assay were kept on ice until they were frozen at the end of each sampling day and kept frozen at -70°C until initial processing and final assay.

Aerosol source sludge samples were collected and processed in conjunction with the aerosol study to be described.

All sludge and sludge-soil samples were kept frozen at -20°C until they were processed. Seven aerosol source sludge samples were examined for virus content.

SECTION 5

RESULTS AND DISCUSSION

EFFICIENCY OF THE $Al(OH)_3$-CONTINUOUS FLOW CENTRIFUGATION TECHNIQUE

During preliminary methodology development, a number of efficiency trials were performed using seeded stock poliovirus type 1 or coxsackievirus B4. For these initial experiments, virus titers were determined by the $TCID_{50}$ endpoint method.(6) As compared to a plaque assay the endpoint method is very imprecise.(21) Further evaluation of the methodology was carried out employing the plaque method for virus assay. Though the first experiments aided in gaining familiarity with the physical aspects of the system, the data was discounted and is not contained herein.

Table 2 shows the results of efficiency studies performed employing the aluminum hydroxide-continuous flow centrifuge technique and the plaque assay method. Three different water sources were employed in the trials. Four of the trials were run with a relatively high seed of poliovirus type 1 (7.8×10^5 pfu - 4.9×10^7 pfu/sample) and one was run with a much lower virus seed (3.6×10^3 pfu/sample).

The apparent recoveries of greater than 100% may have resulted from disaggregation of virus clumps during the experimental manipulations and/or from the experimental error associated with the virus assay.

ISOLATION OF VIRUSES AND BACTERIA FROM WATER SAMPLES

A total of 68 water samples were processed for virus isolation employing the aluminum hydroxide techniques. Tables 3 through 8 list the samples, the bacteriological data and the virological data obtained for each sampling date throughout the study. Concentration factors for water samples ranged from approximately 100 to 1000 fold depending upon the amount of particulate matter suspended in the original sample. Many of the R-3, S-1 and S-2 samples contained soil, algae and other particulate matter. Well water samples usually contained large amounts of iron oxide. Runoff trough and runoff basin waters contained soil and sludge particles.

TABLE 2. EFFICIENCY OF $Al(OH)_3$-CONTINUOUS FLOW CENTRIFUGATION TECHNIQUE AS DETERMINED FROM FULTON COUNTY WATER SAMPLES SEEDED WITH POLIOVIRUS TYPE 1

Sample Source	Sample Volume (ml)	Total Seeded Virus (pfu)	Virus in Concentrate (pfu/ml)	Concentrate Volume (ml)	Total Virus Recovered (pfu)	Recovery Efficiency %
R-3[a]	3,000	7.8×10^5	6.0×10^4	44	2.6×10^6	333
S-1[a]	10,000	4.9×10^7	8.6×10^6	18	1.5×10^8	306
W-12[a]	10,000	4.9×10^7	1.1×10^6	34	3.7×10^7	76
R-3[a]	10,400	4.9×10^7	6.0×10^6	10	6.0×10^7	122
R-3[b]	5,000	3.6×10^3	1.4×10^5	60	2.4×10^3	67

[a]Virus assay in BGM cell cultures.

[b]Virus assay in Hep 2 cell cultures.

Reservoir R-3 drains much of the area that is sludge treated. Big Creek also drains much of the treated area. Sampling stations S-1 and S-2 are respectively the points where Big Creek enters and exits the MSDGC property: Reservoir R-10 does not receive any runoff from sludge treated fields. Basin B-13-1 in a runoff basin that receives runoff directly from a sludge treated field (Field 13).

Statistical comparisons of bacteriological data from surface waters (Table 3), runoff basin water (Table 7), and reservoir R-10 (Table A1, data from MSD Log books) are presented in Appendix A. These comparisons establish relationships among the various types of surface waters as follows:

> The bacterial levels (TC, FC, and FS) in a reservoir (R-3 receiving runoff from sludge treated fields, are higher than the bacterial levels in a reservoir (R-10) receiving runoff from fields receiving no sludge.
>
> Basin runoff water (B-13-1) (see Table 7) is higher in total coliforms than is the water from R-3; while there is no difference between the two in the levels of fecal coliforms and fecal streptococcus. This is not considered a degradation of water quality since in Illinois the only criterion is the fecal coliform count.
>
> A ranking of B-13-1, R-3, and R-10 with respect to some bacterial indicator organisms is possible, with the levels of total coliform and fecal streptococcus increasing in the order R-10, R-3, and B-13-1. There is no statistical difference among the surface water types with respect to fecal coliform.

Comparisons between R-3 and R-10 are difficult to make because of the difference in size between the two and the size of the area drained by each reservoir. R-3 is a large reservoir formed by damming Evelyn Creek while R-10 is a strip-mine lake. It is more productive to compare R-10 with a similar strip-mine lake, R-12, which drains a sludge application area. Recent information (22) indicates that R-12 and R-10 are virtually indistinguishable in terms of indicator organisms.

Although one can rank B-13-1, R-3, and R-10 in terms of some indicator organisms (see above) it is difficult to interpret this ranking with respect to water quality. The water in B-13-1 is runoff from an adjacent field to which sludge has been applied. At any given time various indicator counts may be higher than those in R-3 and R-10. This runoff water is not released, however, until the FC counts are less than 494/100ml. This value is set by the Illinois Environmental Protection Agency and serves to protect the reservoirs receiving the runoff from a deterioration in water quality.

TABLE 3. BACTERIOLOGICAL DATA FROM THE FULTON COUNTY SURFACE WATER ANALYSIS

Sample Source	Date	Bacteria (cfu/l) Total Coliform	Fecal Coliform	Fecal Streptococcus
S-1	8-8-75	2.0×10^6	1.2×10^5	2.3×10^4
S-2	8-8-75	1.5×10^5	1.7×10^4	3.2×10^3
R-3	8-8-75	2.0×10^3	4.0×10^2	1.0×10^2
S-1	9-22-75	6.0×10^5	9.0×10^4	1.4×10^3
S-2	9-22-75	4.3×10^5	2.8×10^4	2.3×10^3
R-3	9-22-75	6.0×10^3	2.0×10^2	7.0×10^3
S-1	10-23-75	7.5×10^4	4.0×10^3	2.5×10^3
S-2	10-23-75	6.0×10^3	1.0×10^2	4.5×10^3
R-3	10-23-75	2.3×10^5	$<1.0 \times 10^2$	$<1.0 \times 10^2$
S-1	11-5-75	NA	NA	NA
S-2	11-5-75	NA	NA	NA
R-3	11-5-75	NA	NA	NA
S-1	12-10-75	3.1×10^6	3.9×10^5	5.0×10^3
S-2	12-10-75	1.0×10^3	1.0×10^3	1.0×10^2
S-1	1-14-76	1.5×10^6	$>6.0 \times 10^3$	1.7×10^4
S-2	1-14-76	2.3×10^6	2.3×10^4	1.8×10^3
R-3	1-14-76	$<1.0 \times 10^3$	$<1.0 \times 10^2$	2.0×10^2
S-1	2-5-76	1.9×10^5	5.1×10^3	2.1×10^3
S-2	2-5-76	NA	2.2×10^3	5.0×10^2
R-3	2-5-76	2.4×10^4	1.0×10^2	1.0×10^2
S-1	3-4-76	4.2×10^6	1.3×10^5	8.6×10^4
S-2	3-4-76	1.8×10^6	2.3×10^5	7.0×10^4
R-3	3-4-76	1.2×10^2	1.0×10^2	1.0×10^2
S-1	4-8-76	3.8×10^5	1.9×10^4	3.8×10^3
S-2	4-8-76	1.2×10^5	3.1×10^4	4.0×10^2
R-3	4-8-76	9.0×10^3	$<2.0 \times 10^1$	$<5.0 \times 10^1$

(continued)

TABLE 3 (continued)

Sample Source	Date	Bacteria (cfu/l) Total Coliform	Total Coliform	Fecal Streptococcus
S-1	5-13-76	1.0×10^5	2.2×10^4	1.0×10^5
S-2	5-13-76	1.0×10^5	1.9×10^4	6.0×10^3
R-3	5-13-76	$< 2.0 \times 10^1$	$< 5.0 \times 10^1$	$< 5.0 \times 10^1$
S-1	6-16-76	1.9×10^5	5.6×10^3	1.8×10^3
S-2	6-16-76	9.0×10^4	3.6×10^3	1.0×10^3
R-3	6-16-76	2.0×10^3	1.0×10^2	3.0×10^2
S-1	7-14-76	1.5×10^4	3.9×10^3	2.4×10^3
S-2	7-14-76	6.0×10^2	$< 5.0 \times 10^1$	2.4×10^3
R-3	7-14-76	5.0×10^2	1.0×10^2	3.5×10^3
S-1	7-21-76	1.2×10^5	1.5×10^4	1.1×10^3
S-2	7-21-76	1.4×10^4	6.0×10^3	1.8×10^3
S-1	8-5-76	1.7×10^5	7.5×10^3	9.1×10^3
S-2	8-5-76	4.9×10^4	6.0×10^3	7.5×10^3
R-3	8-5-76	5.8×10^2	1.6×10^2	6.6×10^2
S-1	9-14-76	5.1×10^5	1.5×10^4	2.1×10^3
S-2	9-14-76	6.5×10^4	5.2×10^3	4.0×10^3
R-3	9-14-76	8.4×10^2	1.6×10^2	8.0×10^1

NA - Lab error - Data not available.

TABLE 4. VIROLOGICAL DATA FROM FULTON COUNTY SURFACE WATER ANALYSIS

Sample Source	Date	Sample Volume (liters)	Concentrate Volume (ml)	Concentrate Volume Assayed (ml)	Number of Plaques Observed	pfu/l[a]	Plaques Confirmed / Picked	Number of Plaques Identified (Identity)	Viruses/l
S-1	8/8/75	4.0	25	25	8	2.0	b	c	--
S-2	8/8/75	4.0	31	31	5	1.25	b	c	--
R-3	8/8/75	20.0	27	27	0	--	--	--	--
S-1	9/22/75	4.0	33	33	4	1.0	b	c	--
S-2	9/22/75	4.0	40	40	12	3.0	b	c	--
R-3	9/22/75	20.0	35	35	6	0.3	b	c	--
S-1	10/23/75	8.0	66	56	38	5.6	10/10	all P1	5.6 d
S-2	10/23/75	6.0	40	30	348	77.6	10/10	all P1	77.6 d
R-3	10/23/75	18.2	50	40	46	3.2	10/10	all P1	3.2 d
S-1	11/5/75	6.0	20	10	12	4.0	2/2	e	4.0
S-2	11/5/75	8.0	50	40	0	--	--	--	--
R-3	11/5/75	16.0	45	35	0	--	--	--	--
S-1	12/10/75	1.7	20	12	0	--	--	--	--
S-2	12/10/75	4.0	20	15	0	--	--	--	--
S-1	1/14/76	6.6	22	17	1	0.20	1/1	(echo 1)	0.2
S-2	1/14/76	7.6	30	25	2	0.32	1/2	e	0.2
R-3	1/14/76	18.2	30	25	0	--	--	--	--
S-1	2/5/76	7.8	26	20	0	--	--	--	--
S-2	2/5/76	7.4	29	29	1	0.14	0/1	--	--
R-3	2/5/76	18.0	42	37	1	0.06	0/1	--	--

(continued)

TABLE 4. (continued)

Sample Source	Date	Sample Volume (liters)	Concentrate Volume (ml)	Concentrate Volume Assayed (ml)	Number of Plaques Observed	pfu/l[a]	Plaques Confirmed / Picked	Number of Plaques Identified (Identity)	Viruses/l
S-1	3/4/76	7.6	48	42	3	0.35	0/3	--	--
S-2	3/4/76	7.7	47	44	1	0.12	0/1	--	--
R-3	3/4/76	17.2	35	30	1	0.07	0/1	--	--
S-1	4/8/76	7.5	48	40	1	0.16	0/1	--	--
S-2	4/8/76	7.6	36	30	0	--	--	--	--
R-3	4/8/76	18.7	50	45	0	--	--	--	--
S-1	5/13/76	7.8	36	31	0	--	--	--	--
S-2	5/13/76	7.9	38	33	0	--	--	--	--
R-3	5/13/76	18.5	32	27	0	--	--	--	--
S-1	6/16/76	7.8	34	29	0	--	--	--	--
S-2	6/16/76	7.7	34	29	0	--	--	--	--
R-3	6/16/76	15.6	33	28	0	--	--	--	--
R-3	7/14/76	20.4	18	15	0	--	--	--	--
S-1	7/21/76	20.0	32	25	0	--	--	--	--
S-2	7/21/76	20.0	39	34	0	--	--	--	--
S-1	8/5/76	19.8	40	35	0	--	--	--	--
S-2	8/5/76	20.5	38	33	0	--	--	--	--
R-3	8/5/76	20.2	25	25	0	--	--	--	--

(continued)

TABLE 4. (continued)

Sample Source	Date	Sample Volume (liters)	Concentrate Volume (ml)	Concentrate Volume Assayed (ml)	Number of Plaques Observed	pfu/l[a]	Plaques Confirmed / Picked	Number of Plaques Identified (Identity)	Viruses/l
S-1	9/14/76	20.2	27	22	0	--	--	--	--
S-2	9/14/76	20.6	26	21	0	--	--	--	--
R-3	9/14/76	20.0	21	16	0	--	--	--	--

a. Calculated from number of plaques counted.

b. No plaques were confirmed.

c. No plaques were identified.

d. Laboratory contamination - see text.

e. Isolates were neutralized by all antisera in pools.

TABLE 5. BACTERIOLOGICAL DATA FROM THE FULTON COUNTY WELL WATER ANALYSIS

Sample Source	Date	Bacteria (cfu/l) Total Coliform	Fecal Coliform	Fecal Streptococcus
W-14	10-30-75	NA	NA	NA
W-14	11-20-75	NA	NA	NA
W-14	12-18-75	$<1.0x10^3$	$<1.0x10^2$	$<1.0x10^2$
W-6	12-18-75	NA	NA	NA
W-14	1-6-76	$1.0x10^2$	$<1.0x10^2$	$<1.0x10^2$
W-12	1-22-76	$8.0x10^2$	$<1.0x10^2$	$<1.0x10^2$
W-12	2-9-76	$<1.0x10^3$	$<1.0x10^2$	$<1.0x10^2$
W-12	3-16-76	$<1.0x10^2$	$<1.0x10^2$	$<1.0x10^2$
W-14	3-18-76	$<1.0x10^2$	$<1.0x10^2$	$<1.0x10^2$
W-12	3-30-76	$<1.0x10^2$	$<1.0x10^2$	$<1.0x10^2$
W-12	4-13-76	NA	$<1.0x10^2$	NA
W-6	4-27-76	NA	$<1.0x10^2$	NA
W-12	5-20-76	$<5.0x10^1$	$<5.0x10^1$	$<5.0x10^1$
W-12	7-7-76	$<5.0x10^1$	$<5.0x10^1$	$1.0x10^2$
W-12	7-22-76	$1.0x10^2$	$<1.0x10^1$	$<1.0x10^1$
W-12	9-8-76	$<2.0x10^1$	$<2.0x10^1$	$<2.0x10^1$
W-14	9-22-76	$1.6x10^2$	$<2.0x10^1$	$5.2x10^2$

NA - Lab error - Data not available

TABLE 6. VIROLOGICAL DATA FROM FULTON COUNTY WELL WATER ANALYSIS

Sample Source	Date	Sample Volume (liters)	Concentrate Volume (ml)	Concentrate Volume Assayed (ml)	Number of Plaques Observed	pfu/l[a]	Plaques Confirmed / Picked	Number of Plaques Identified (Identity)	Viruses/l
W-14	10-30-75	40	120	110	696	19.0	0/9	--	--
W-14	11-20-75	20	40	35	2	0.1	0/2	--	--
W-14	12-18-75	30	70	43	0	--	--	--	--
W-6	12-18-75	40	20	14	0	--	--	--	--
W-14	1-6-76	20	80	65	0	--	--	--	--
W-12	1-22-76	20	38	34	0	--	--	--	--
W-12	2-9-76	16	45	40	0	--	--	--	--
W-12	3-16-76	20	65	55	4	0.22	0/2	--	--
W-14	3-18-76	20	44	40	2	0.11	0/2	--	--
W-12	3-30-76	39.6	79	70	0	--	--	--	--
W-12	4-13-76	40.4	78	70	0	--	--	--	--
W-6	4-27-76	39.9	42	39	6	0.16	0/6	--	--
W-12	5-20-76	40.2	78	73	0	--	--	--	--

(continued)

TABLE 6. (continued)

Sample Source	Date	Sample Volume (liters)	Concentrate Volume (ml)	Concentrate Volume Assayed (ml)	Number of Plaques Observed	pfu/l[a]	Plaques Confirmed / Picked	Number of Plaques Identified (Identity)	Viruses/l
W-12	7-7-76	40.0	40	35	0	0			0
W-12	7-22-76	40.4	64	59	0	0			0
W-12	9-2-76	38.7	47	42	0	0			0
W-12	9-8-76	35.0	45	40	0	0			0
W-14	9-22-76	39.2	38	33	0	0			0

a. Calculated from number of plaques observed.

TABLE 7. BACTERIOLOGICAL DATA FROM THE FULTON COUNTY RUNOFF WATER ANALYSIS

Sample Source	Date	Bacteria (cfu/l)		
		Total Coliform	Fecal Coliform	Fecal Streptococcus
B-13-1	11-12-75	4.0×10^4	5.0×10^2	3.5×10^3
B-13-1	11-18-75	3.7×10^5	1.0×10^2	1.4×10^3
B-13-1a	12-3-75	1.0×10^3	$<1.0 \times 10^2$	1.2×10^4
B-13-1b	12-3-75	7.0×10^4	8.0×10^2	2.7×10^4
B-13-1c	12-3-75	5.2×10^4	1.3×10^3	1.8×10^5
RT-13	2-23-76	3.0×10^2	$<1.0 \times 10^2$	4.0×10^2
RT-13	8-4-76	1.9×10^4	2.0×10^2	1.3×10^4
B-13-1	9-22-76	7.0×10^3	$<1.0 \times 10^2$	5.7×10^3

TABLE 8. VIROLOGICAL DATA FROM FULTON COUNTY RUNOFF WATER ANALYSIS

Sample Source	Date	Sample Volume (liters)	Concentrate Volume (ml)	Concentrate Volume Assayed (ml)	Number of Plaques Observed	pfu/l	Plaques Confirmed / Picked	Number of Plaques Identified (Identity)	Viruses/l
B-13-1	11-12-75	2.0	40	20	0	--	--	--	--
B-13-1	11-18-75	8.0	60	50	0	--	--	--	--
B-13-1a	12-3-75	7.6	20	10	0	--	--	--	--
B-13-1b	12-3-75	6.9	56	46	0	--	--	--	--
B-13-1c	12-3-75	7.5	40	29	0	--	--	--	--
RT-13	2-23-76	13.0	36	30	0	--	--	--	--
RT-13	8-4-76	3.9	17	15	a	--	--	--	--
B-13-1	9-22-76	7.8	26	10	0	--	--	--	--

a Sample was toxic to cell cultures.

The stream sampling stations S-1 and S-2 (see Figure 2, and Table 3) were chosen to reflect the quality of the water in a stream at the point of entry and exit, respectively, from the MSDGC property. With respect to any given parameter then, stations S-1 and S-2 would be expected to reflect the effects of sludge application activities on the stream.

Statistical comparisons between station S-1 and S-2 (see Appendix A) indicate that the levels of total coliforms at S-2 are significantly lower than at S-1. There do not appear to be any differences in fecal coliform or fecal streptococcus levels between the two stations. These comparisons indicate that the sludge application to the MSDGC property is not resulting in a decrease in water quality in the stream (Big Creek) which drains the majority of the sludge application area. Some consideration must be given to the flow patterns at the two stream sampling sites (S-1 and S-2) and at R-3 which discharges into Evelyn Creek. The mean flows at S-1, S-2, and R-3 during the 1976 water year were 22.7, 32.2, and 3.64 cubic feet per second (cfs) respectively. The flow at R-3 represents only 38% of the difference in flow between S-1 and S-2, thus 72% of this difference comes from stormwater runoff, seepage and other non-gaged streams or sources which are not monitored for indicator organisms. The actual conditions, therefore, seem to rule out erosion of stream quality due to the sludge application activities of the MSDGC and are consistent with the statistical comparisons.

No viruses could be detected in any well samples (Table 6). No statistical analysis of bacteriological data for well water (Table 5) was attempted due to the indeterminate nature of the data.

Samples collected on October 23, 1975 from S-1, S-2, and R-3 yielded viruses (Table 4) that were identified as poliovirus type 1 by serum neutralization tests. They were identified as vaccine strain by temperature sensitivity tests. A 3 to 4 log reduction in titer was noted when virus was propagated at 40°C compared to 37°C. Subsequent studies indicated that these isolates were laboratory contaminants.

An evaluation of the data in Table 4 shows that the number of poliovirus 1 isolates in the S-1, S-2, and R-3 samples of 10/23/75 is striking. Since we had performed an efficiency study on 10/22/75 by inoculating 7.8×10^5 pfu poliovirus 1 into 3 liters of R-3 water, we felt there was a source of viral contamination in the concentration system itself. Thus an efficiency study was repeated in which 10 liters of W-12 water were seeded with 4.9×10^7 pfu poliovirus 1. The following day, 39 liters of unseeded W-12 water were processed and 7.2×10^3 pfu poliovirus 1 was recovered, indicating a definite carryover of virus in the centrifugation system. In an attempt to pinpoint the source

of contamination, 1 ml of approximately 6 x 10^6 pfu poliovirus 1 was added to 3 liters of tap water, then processed. The processing equipment was dismantled and disinfected as usual. With the exception of the flowmeter, all parts were soaked overnight in 1; 1000 Roccal, then soaked and washed in hot water containing 7X cleaning solution (Linbro), rinsed twice in tap water and once in double distilled water and dried. The equipment was reassembled and sterile saline added. No virus was found in the centrifuge head. However, 5.5 x 10^3 pfu poliovirus was found in the tubing. Untreated fluids in the flowmeter at the inflow position contained more than 10^2 pfu virus after 24 hours at room temperature. As a result, after December, 1975 and for the duration of the program, all parts were autoclaved or, as in the case of the rubber tubing, replaced to rectify this problem. In addition, the flowmeter was changed to an outflow position. These data point out the need for extreme caution when decontaminating equipment to be employed in these types of environmental studies.

Assay of water concentrates from site S-1 (1-14-76) yielded an echovirus type 1 as identified by serum neutralization tests. Two other confirmed isolates from S-1 (11/5/75) and S-2 (1/14/76) could not be completely identified. Both 3.2 x 10^2 and 3.2 x 10^4 $TCID_{50}$ of these isolates were totally neutralized by 25 antibody units of all eight of the Lim Benyesh-Melnick serum pools. On the other hand, they were not neutralized by monospecific poliovirus 1, 2, and 3 or echovirus 9, 12, and 21 antisera. Both isolates were 0.2 um in size as determined by their passage through a Nuclepore 0.2 um filter. They were ether and acid stable and negative for mycoplasma. The isolates could be enteroviruses; adenoviruses or reoviruses.

Plaque-like lesions were also observed during assay of 14 other samples. These lesions varied in size and shape, some best described as "bulls eye" type or central stained cells surrounded by a circle of unstained cells surrounded by stained cells. All areas were monitored microscopically to assure that confluent monolayers were present beneath the agar overlay. These lesions were picked and blind-passaged twice in fluid BGM and/or RD cell cultures. Inasmuch as CPE was not observed in either passage during 14 days of incubation, these samples were considered negative for viruses.

Table 9 summarizes the data from the virus isolation studies. Six of the 68 sample concentrates were found to contain virus which could be subpassaged in cell culture, titered and identified. Three of these six positive samples (S-1, S-2 and R-3 all from the 10/23/75 sampling date) were determined to be contaminants and contained poliovirus 1, Sabin strain. The other three positive samples (S-1, 11/5/75; S-1 and S-2, 1/14/76) contained virus other than the poliovirus 1, Sabin strain.

TABLE 9. FREQUENCY OF VIRUS ISOLATION FROM WATER SAMPLES

Sample Source	No. of Samples Positive for Virus/ No. of Samples	Virus Isolates	No. of Samples Positive for Virus Due to Cross-contamination[a]/ No. of Samples	Virus Isolates Identified
S-1	2/15	Echovirus 1 Unidentified Isolate	1/15	Poliovirus 1, Vaccine Strain
S-2	1/15	Unidentified Isolate	1/15	Poliovirus 1, Vaccine Strain
R-3	0/12		1/12	Poliovirus 1, Vaccine Strain
W-12	0/9		0/9	
W-14	0/6		0/6	
W-6	0/2		0/2	
B-13-1	0/6		0/6	
RT-13	0/2		0/2	
Total	3/68		3/68	

[a] Contamination of continuous-flow centrifugation tubing - see text.

EVALUATION OF POLYETHYLENE GLYCOL HYDROEXTRACTION TECHNIQUE FOR CONCENTRATING VIRUSES FROM SLUDGE

Different types of sludge samples were seeded with poliovirus 1, Sabin strain, 9.46×10^6 to 2.24×10^7 pfu and processed for virus concentration using the polyethylene glycol (PEG) hydroextraction technique. As shown in Table 10, virus recoveries ranged from 13.7% to 44.4% in a sludge-soil mixture and anaerobic digester draw-off I samples, respectively. In other attempts to evaluate the methodology, the virus recoveries were 19.9% and 21.6% in sludge samples from holding basin and anaerobic digester draw-off II, respectively.

DETECTION OF INDIGENOUS VIRUSES IN SLUDGE SAMPLES

Sludge samples from the field pilot sludge lagoon, sludge which was incorporated into field 17 sediment from runoff basin 13-1 and sludge applied via spray application were processed and assayed for viruses.

Virus Survival in Sludge Lagoon

A pilot lagoon (41m x 27m x 6m) was constructed and filled from the same sludge source as the full scale lagoons existing at the Fulton County site. The purpose of this study was to determine what quantities of virus if any, are present in such sludge and also to monitor the survival of viruses in lagooned sludge. As shown in Table 11, no virus was detected in either supernatant or sediment samples collected over a two month period. Since no virus was detected in either supernatants or sediments for 6 sampling periods (4 initial consecutive sampling and 2 later consecutive sampling periods); efforts to isolate viruses from this source were discontinued.

Determination of Viruses in Sludge Incorporated Fields and Basin 13-1 Samples

Two hundred gram sludge-soil samples from a sludge incorporated field were processed to viruses to 13-15 ml of final volume and were assayed in BGM cell cultures. Plaque-like lesions (28-40) were observed in each sample (Table 12). One half of the plaques from each sample were picked and passaged in BGM tube cultures. None of the plaques induced cytopathogenic effect on second passage in BGM cells. These results indicated that the plaques were produced by "non-viral" material. Similarly, when 200-400 ml of sludge samples from runoff basin 13-1 were concentrated and assayed for plaques, none of the "plaques" which developed could be confirmed as viruses upon subpassage (Table 12).

TABLE 10. EFFICIENCY OF POLYETHYLENE GLYCOL HYDROEXTRACTION TECHNIQUE FOR CONCENTRATING VIRUSES FROM DIFFERENT SLUDGE SAMPLES[a]

Sample Type	Volume (ml) Initial	Volume (ml) Final	Poliovirus Seeded pfu	Poliovirus Recovered pfu	Percent Efficiency
Anaerobic Digester Draw-off I	200	45	1.62×10^7	7.2×10^6	44.4
Fulton County Sludge Holding Basin	200	35	1.62×10^7	3.22×10^6	19.9
Anaerobic Digester Draw-off II	200	45	2.24×10^7	4.85×10^6	21.6
Fulton County Sludge Incorporate Field (Sludge-soil mixture)	200	25	9.46×10^6	5.2×10^4	13.7

[a] virus assays run in BGM cell cultures.

TABLE 11. VIROLOGICAL EXAMINATION OF FULTON COUNTY SLUDGE LAGOON SAMPLES

Sample Number	Date	Concentrated Volume* (ml)		Number of Plaques	
		Supernatant	Sediment	Supernatant	Sediment
01-S1	6-28	12	20	--	--
02-SL	7-15	18	10	--	--
03-SL	7-23	35	18	--	--
04-SL	7-29	14	19	--	--
08-SL	8-25	24	15	--	--
09-SL	9-2	12	25	--	--

* Initial volume of all supernatant and sediment samples was 200 ml.

TABLE 12. VIROLOGICAL EXAMINATION OF FULTON COUNTY SLUDGE INCORPORATED FIELD AND RUNOFF BASIN 13-1 SAMPLES

Sample Number	Date	Sample Size		Number of Plaques	Plaques Confirmed
		Initial	Final		
*					
01-IP	7-19-76	200 g	15 ml	34	0
02-IP	8-3-76	200 g	15 ml	28	0
03-IP	8-18-76	200 g	13 ml	40	0
**					
01-B13	6-21-76	200 ml	50 ml	29	0
02-B13	9-9-76	200 ml	26 ml	4	0
03-B13	10-14-76	400 ml	52 ml	58	0
04-B13	10-22-76	400 ml	51 ml	17	0

* IP - Sludge-soil mixture samples from sludge incorporated field.

** B13 - Bottom sediment samples from Runoff holding basin 13-1.

Employing the sludge virus isolation system consisting of hydro-extraction concentration and BGM assay, no viruses were isolated from the sludge examined.

Virus Detection in Sludge Applied Via Spraying

Seven different sludge samples (200 ml each) from the spray application system were concentrated and assayed for viruses as described. None of the samples were confirmed for viruses (Table 12). Two samples developed 12 plaque-like lesions. Nine of the 12 plaques were blind passaged 3 times. In no case was CPE observed.

As above, employing the virus isolation methodology described, no viruses were confirmed as being present in any of the sludge samples examined.

Virus Movement In Soil - Laboratory Study

Poliovirus type 1 (4.3×10^7 pfu), or echovirus 7 (2.6×10^8 pfu), suspended in sludge or distilled water, were each applied to the surfaces of five columns containing soil. The columns were constructed of plexiglass and each contained 3.5 kg of Fulton County soil, sieved to exclude particles greater than 2 mm in diameter. Prior to the application of viruses in water or sludge, the columns were saturated with distilled water, and then 100 ml of distilled water was added to each column daily for one to two weeks until the eluate volume stabilized at from 75-95 ml per day. The balance was lost by evaporation.

For poliovirus type 1, 100 ml of digested sludge seeded with 4.3×10^7 pfu were applied to three (3) columns. Two other columns were treated with 100 ml of unseeded digested sludge, and 100 ml of distilled water seeded with 4.3×10^7 pfu, respectively. Echovirus 7, 2.6×10^8 pfu in distilled water or sludge, was applied to similar soil columns. Immediately after addition of the sludge, or distilled water with or without virus, the contents of one column containing sludge and seeded virus was completely mixed and assayed for virus concentration and dry weight. The recoveries of poliovirus 1 and echovirus 7 immediately after mixing were 70 & 8% respectively (see Table 13). This loss may reflect the method used to recover the viruses from the soil.

Distilled water was added to each remaining column at a rate of 100 ml per day for five (5) days. This is equivalent to a rainfall of 1.70 in over five (5) days. One (1) ml of eluate was assayed daily for virus assay. After five (5) days, in each experiment, a column containing seeded sludge was thoroughly

mixed, as described, and assayed for virus and dry weight. In addition, after five (5) days, a seeded sludge column was frozen solid, (in a freezer), and the plexiglass casing removed. Aliquots of soil were taken with a 3/8" cork borer from approximate depths of 1.3 cm, 2.6 cm, 5.1 cm, 10.2 cm, 20.4 cm and the bottom of the soil columns. No soil samples were assayed for viruses from the columns receiving unseeded sludge.

The results of these soil-column studies are shown in Table 13.

No viruses were detected in any of the column eluates from either the poliovirus 1 or the echovirus 7 experimental runs, indicating that the viruses did not penetrate the entire soil column. The initial concentrations of viruses in the thoroughly mixed column were: poliovirus 1, 8.6×10^3 pfu/gram; and echovirus 7, 6.1×10^3 pfu/gram. After five days the virus concentrations in the mixed columns were 1.3 s 10^3 pfu/gram and 1.1×10^3 pfu/gram for poliovirus 1 and echovirus 7 respectively (see Table 13).

If one assumes that the efficiency of recovery of viruses from the soil was the same at the end of the study as at the beginning of the study for the mixed columns, these findings suggest an inactivation over the period of 84.9% and 82.0% for poliovirus 1 and echovirus 7 respectively.

At a depth of 1.3 cm virus concentrations were 1.7×10^3 pfu/gram and 9.1×10^2 pfu/gram for poliovirus 1 and echovirus 7 respectively. In neither case were viruses isolated from depths greater than 1.3 cm (Table 13).

As shown in Table 1 the texture of the soil used in the study was clay loam and had a cation exchange capacity of 14.3 meg/gm. These soil characteristics may have accounted for the minimal virus movement in the columns.

In a recent study in which coxsackievirus type B-3 was added to municipal sludges which were placed on lysimeters containing sandy soil or clay soil, the virus remained bound to sludge placed in the top soil.(23) These findings are in agreement with those presented herein.

AEROSOL STUDIES

Aerosol studies were performed in an effort to assess the microbial contribution to the air downwind of the sprayers. Generally, only one field was receiving sludge by spraying at any particular time. The selection of the field sites for spraying was made by the Maintenance and Operations Department of MSDGC and was not influenced by the presence of the personnel

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TABLE 13

SOIL COLUMN STUDIES: VIRUS PENETRATION AND ADSORPTION BY FULTON COUNTY SOIL - 1976

	Viruses Added to Column pfu	Total 1, 2 Virus Recovered From Column-Start pfu	Total 1, 3 Viruses Recovered From Column-5 Days pfu	Virus Recovered From Eluate pfu/ml					Virus Recovered From Column pfu/gram				
				Time (Days)					Column Depth (cm)				
				1	2	3	4	5	1.3	2.6	5.2	10.4	20.4
Poliovirus Type 1	4.3×10^7	3.0×10^7 (70% Recovery)	4.6×10^6	--	--	--	--	--	1.7×10^3	--	--	--	--
Echovirus7	2.6×10^8	2.1×10^7 (8% Recovery)	3.9×10^6	--	--	--	--	--	9.1×10^2	--	--	--	--

1. Total column value = pfu/gram x 3500 grams/column.
2. Initial virus concentration: Poliovirus 1, 8.6×10^3 pfu/gram; echovirus 7, 6.1×10^3 pfu/gram.
3. Final virus concentration: Poliovirus 1, 1.3×10^3 pfu/gram; echovirus 7, 1.1×10^3 pfu/gram.

engaged in the aerosol studies.

These studies were performed during three separate sampling periods: the weeks of May 24, July 19, and August 30, 1976. Conditions during each of these sampling periods were varied in wind direction and velocity, temperature and relative humidity. Wind velocity ranged from 0.5 to 4.3 meters per second, temperature from 20.1 to 30.6°C, and relative humidity from 31% to 87%. The July 19 sampling period was restricted by rain, which prevented sludge application during much of that week. The samples collected during that period were preceded by heavy rainfall. Air sampling was performed in the vicinity of the particular field to which sludge was applied by spraying.

Field sampling sites were selected on the basis of accessibility and wind direction relative to the location of the sludge spray gun. During sludge application, the spray gun moved continually, and this resulted in variable distances and locations of the sampling sites relative to the aerosol source. Average estimated distances of sampling locations from the sludge sprayer ranged from 50 to 450 m. The duration of each air sampling period was influenced by sprayer shut down, (resulting from mechanical breakdown or completion of sludge application at a particular site), movement of the sprayer to a location that would not permit representative downwind (or upwind) sampling, or by dramatic shifts in wind direction. Attempts were made to obtain control samples upwind from the spray source. However, it was not always possible to determine the nature of activities that were occurring upwind from a "control" sample. In some cases sludge incorporation activities occurred on fields near the site of sludge spray irrigation but were visually isolated from the site of sampling.

Rough terrain in and surrounding each sludge application field prevented the kind of mobility that would have been necessary to readily readjust sampling positions following significant wind direction alterations. Since periodic shifts in wind direction could not be quantitatively accounted for, reported sampling times should be interpreted as representing maximum periods of downwind sampling.

Table 14 summarizes the results of the aerosol studies. The Litton high-volume sampler (LVAS) indicated bacterial concentrations in the air sampled of 10^4 - 106 cfu/m^3 and for coliphage of 0 - 2.2×10^3 pfu/m^3. These estimates were made at varying distances downwind of the sludge spray apparatus from 50 - 450 meters (see Table 14). Upwind values ranged from 1.5×10^2 - 5.5×10^2 cfu/m^3 for total bacteria and 0 - 1.2×10^2 pfu/m^3 for coliphage, both of these parameters being estimated from LVAS sampler fluid.

METROPOLITAN SANITARY DISTRICT OF GREATER CHICAGO

TABLE 14

CONCENTRATIONS OF ANIMAL VIRUS, COLIPHAGE, AND TOTAL BACTERIA DETECTED IN AEROSOLS AT MSDGC SLUDGE IRRIGATION SITE - MAY, JULY, AUGUST, SEPTEMBER, 1976

Date Sampled	Distance From Source (m)	TEST CONDITIONS: Wind Speed (mi/hr)	Relative Humidity (%)	Temp (^{o}C)	Stability Class	Air Volume Sampled (m^3)	CONCENTRATION/m^3: Animal Viruses (pfu)	Coliphage (mpnpfu)	Total Bacteria (cfu)	Notes
5/25	150	4.9	31	21.1	A-B	30	--	--	$3.1x10^4$	1,5
5/25	400	2.7	31	20.1	A	31	--	--	$2.0x10^5$	1
5/26	50	5.1	39	25.0	B-C	36	--	$1.0x10^{-1}$	$3.8x10^4$	1
5/26	75	4.7	73	22.5	A-B	31	--	--	$1.7x10^4$	1
5/26	100	4.3	72	22.2	A-B	23	--	--	$1.3x10^5$	1
5/26	Upwind Control (450)	4.7	34	22.8	B	30	--	--	$1.5x10^2$	1
5/27	200	4.4	34	24.4	D	40	--	--	$9.9x10^3$	1
5/27	200	4.3	34	23.9	D	49	--	$5.3x10^{-1}$	$4.4x10^4$	1
5/27	250	3.5	78	24.4	D	25	--	--	$2.0x10^4$	1
5/27	300	3.5	78	24.4	D	62	--	$5.5x10^{-2}$	$1.4x10^5$	1
5/27	Upwind Control (*)	3.6	40	22.2	D	30	--	--	$5.5x10^2$	1
5/25	Sludge Spray Source	--	--	--	--	--	--	$1.3x10^3$pfu/l	$8.2x10^8$cfu/l	2,4
5/26	"	--	--	--	--	--	--	$3.0x10^4$pfu/l	$5.2x10^8$cfu/l	2,4
5/27	"	--	--	--	--	--	--	$3.0x10^4$pfu/l	$1.1x10^8$cfu/l	2,4
7/21	50	6.3	66**	28.9	D	37	--	--	--	1
						0.42	--	--	$5.3x10^2$	3
7/21	300	5.6	57	29.4	D	160	--	--	--	1
						1.70	--	--	$5.8x10^1$	3

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TABLE 14 (Cont'd)

CONCENTRATIONS OF ANIMAL VIRUS, COLIPHAGE, AND TOTAL BACTERIA DETECTED IN AEROSOLS AT MSDGC SLUDGE IRRIGATION SITE - MAY, JULY, AUGUST, SEPTEMBER, 1976

Date Sampled	Distance From Source (m)	TEST CONDITIONS				Air Volume Sampled (m^3)	CONCENTRATIONS/m^3			Notes
		Wind Speed (mi/hr)	Relative Humidity (%)	Temp (^oC)	Stability Class		Animal Viruses (pfu)	Coliphage (mpnpfu)	Total Bacteria (cfu)	
7/21	300	4.3	87	25.0	D	31	--	--	--	1
						0.79	--	--	5.2×10^2	3
7/21	Upwind Control (450)	1.6	87	25.8	D	60	***	--	--	1
						1.70	--	--	4.6×10^1	3
7/21	Sludge Spray Source	--	--	--	--	--	--	--	2.8×10^7 cfu/l	2
8/31	200	7.4	47	26.7	B	127	--	3.8×10^{-2}	--	1
						0.56	--	--	6.2×10^2	3
8/31	200	7.2	37	30.6	B	60	2.1×10^{-2}	3.8×10^{-1}	--	1
						0.56	--	--	5.5×10^2	3
8/31	425	4.5	45	27.8	D	175	--	5.5×10^{-2}	--	1
						0.56	--	--	2.2×10^3	3
8/31	450	6.0	43	28.3	D	53	--	5.3×10^{-1}	--	1
						0.56	--	--	6.6×10^3	3
8/31	Upwind Control (150)	6.0	38	29.4	D	127	--	3.9×10^{-2}	--	1
						0.56	--	--	4.2×10^2	3

METROPOLITAN SANITARY DISTRICT OF GREATER CHICAGO

TABLE 14 (Cont'd)

CONCENTRATIONS OF ANIMAL VIRUS, COLIPHAGE, AND TOTAL BACTERIA DETECTED IN AEROSOLS AT MSDGC SLUDGE IRRIGATION SITE - MAY, JULY, AUGUST, SEPTEMBER, 1976

Date Sampled	Distance From Source (m)	TEST CONDITIONS				Air Volume Sampled (m^3)	CONCENTRATIONS/m^3			Notes
		Wind Speed (mi/hr)	Relative Humidity (%)	Temp (^oC)	Stability Class		Animal Viruses (Pfu)	Coliphage (mpnpfu)	Total Bacteria (cfu)	
8/31	Sludge Spray Source	--	--	--	--	--	--	$4.5x10^4$pfu/l	$2.2x10^7$cfu/l	2
9/1	50	9.6	69	23.3	D	10	--	$2.2x10^3$pfu	--	1
						0.53	--	--	$1.4x10^3$	3
9/1	150	3.1	47	26.7	A	36	--	$4.7x10^2$pfu	$1.7x10^5$	1,4
9/1	150	7.6	47	28.3	D	26	$1.0x10^{-1}$	$6.8x10^2$pfu	$1.1x10^6$	1,4
9/1	175	7.6	53	27.8	D	69	--	$4.2x10^2$pfu	--	1,4
						0.56	--	--	$8.5x10^2$	3
9/1	400	3.1	47	26.7	A	66	--	$1.8x10^{-1}$pfu	$6.8x10^5$	1,4
9/1	Sludge Spray Source	--	--	--	--	--	--	$7.0x10^4$pfu/l	$2.8x10^9$cfu/l	2
9/2	350	4.9	N.A.	27.8	A-B	206	$6.7x10^{-3}$	$6.4x10^1$pfu	--	1,4
						0.56	--	--	$4.9x10^2$	3

METROPOLITAN SANITARY DISTRICT OF GREATER CHICAGO

TABLE 14 (Cont'd)

CONCENTRATIONS OF ANIMAL VIRUS, COLIPHAGE, AND TOTAL BACTERIA DETECTED IN AEROSOLS AT MSDGC SLUDGE IRRIGATION SITE - MAY, JULY, AUGUST, SEPTEMBER, 1976

Date Sampled	Distance From Source (m)	TEST CONDITIONS: Wind Speed (mi/hr)	Relative Humidity (%)	Temp (^{o}C)	Stability Class	Air Volume Sampled (m^3)	CONCENTRATIONS/m^3: Animal Viruses (pfu)	Coliphage (mpnpfu)	Total Bacteria (cfu)	Notes
9/2	Upwind Control (175)	2.7	N.A.	25.0	A	63 0.56	4.2×10^{-2} --	1.2×10^{2}pfu --	-- 3.6×10^{2}	1,4 3
9/2	Sludge Spray Source	--	--	--	--	--	--	6.0×10^{4}pfu/l	1.2×10^{10}cfu/l	2

1. Counts from Litton High-Volume sampler fluid. 0 = no positive observation.
2. 200 ml spray source concentrated for animal virus counts. For coliphage counts, mpn tubes scored positive by placing drop from tube on "lawn" of host cells E. coli C3000, and observing for plaque formation bacterial counts by serial dilution and spread plates on trypticase soy agar.
3. Bacterial counts by Anderson six-stage sampler using trypticase soy agar.
4. Coliphage counts by direct plaque assay using serial dilutions and soft agar overlays with host cells E. coli C3000.
5. All stability classes based on method of Lighthart and Frisch (20).

* Sample collected on premises of R&D Laboratory with sludge incorporation ongoing in an adjacent field.

** Trace precipitation during collection period.

*** Sample lost during processing.

Total colony forming units (cfu) estimated with the Andersen six-stage sampler ranged from $4.6 \times 10^1 - 3.6 \times 10^2$ cfu/m^3 for upwind control samples and from $5.8 \times 10^1 - 6.6 \times 10^3$ for samples taken downwind at varying distances (see Table 14).

Sludge spray source samples were also examined for the viral and bacterial parameters. No animal virus were found in any of the spray source samples, while coliphage and total bacteria ranged from $0 - 7.0 \times 10^4$ pfu/l and $2.2 \times 10^7 - 1.2 \times 10^{10}$ cfu/l respectively.

The data from 24 Andersen six-stage samplers was pooled to provide an estimate of the size distribution of the particles captured by these instruments. The greatest percentage of viable particles captured was in the 9.2 um size range. A cumulative average of approximately 40% of the particles collected were in the 5.5 um or less size range (see Figure 3). Within this size range the highest percentage of viable particles was between 1.0 - 2.0 um (see Figure 3).

It can be seen from the data in Table 14 that the combination of environmental variables which can be expressed as stability class (Lighthart & Frisch, (20)) had little or no effect on the recovery of coliphage or bacteria whether sampled with the high-volume sample or the Andersen six-stage impactor.

Although animal viruses were not detected in the sludge spray source, 4 ambient air samples had detectable virus levels. Of these 4 samples one was an upwind (control) sample.

A total of six plaques were confirmed (on second or third blind passage) as animal virus. Each of these viruses was identified as poliovirus type 1. Akin and Jakubowski (24) have discussed the problems of contamination which threaten this type of work, and since all of the viruses identified in the ambient air samples were of the same type (poliovirus 1) as that used for experiments conducted simultaneously in the same laboratory this possibility cannot be dismissed.

All of the available data for animal virus isolations, total bacteria, coliphage and the environmental information shown in Table 14, was subjected to rigorous statistical analysis.

This statistical analysis is presented in Appendix B. From this analysis the following inferences may be drawn:

1. The presence of animal virus downwind of the sludge spray source during active application periods is not independent of the background conditions. There is evidence which indicates that the spray application is not the only source of virus.

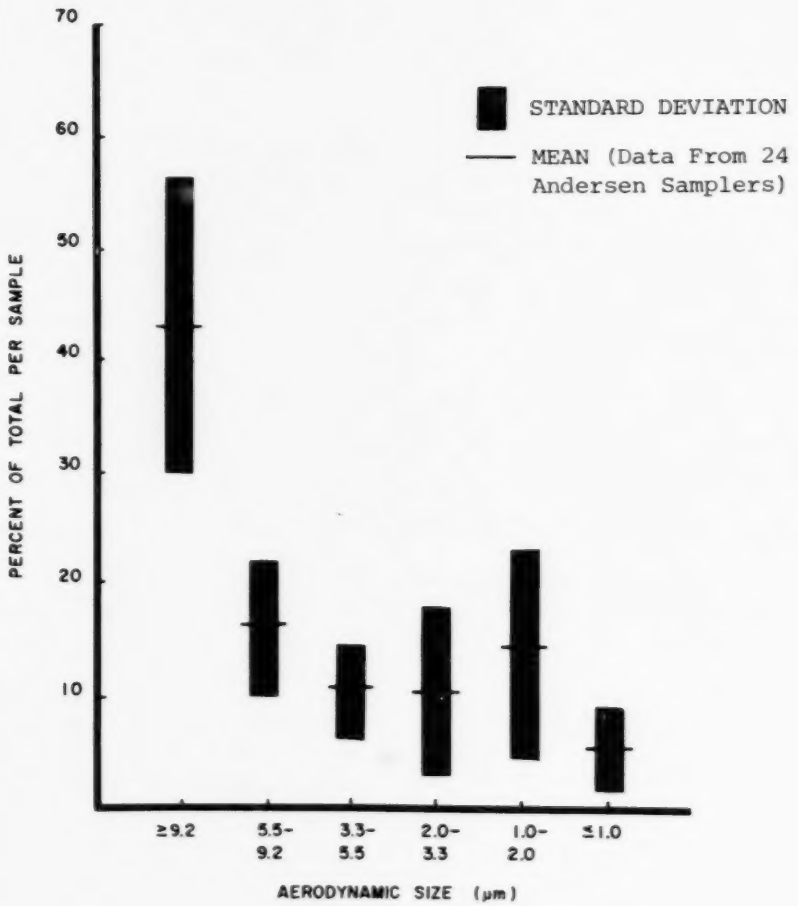

Figure 3. Aerodynamic size of total bacteria - containing particles

2. The levels of coliphage decrease exponentially with distance of the sampler from the spray source.

3. The Andersen six-stage sampler and the Litton high-volume sampler provide different estimates for total bacterial counts.

4. Wind velocity, temperature and relative humidity seem to have little influence on downwind concentrations of the coliphage and bacterial parameters.

5. If one assumes that the sludge spray source remains constant, then temperature becomes a determining factor in the levels of total bacteria captured in aerosols downwind.

Some discussion of these results is in order in light of previous work with microbial aerosols.

A considerable amount of research effort has been expended in measuring microbial aerosols emanating from wastewater treatment plants of various types.(25) Although these studies do not exactly correspond to what we have attempted here they are instructive for what has been accomplished.

Only two later studies have actually tried to determine the presence of animal viruses in the aerosol from wastewater reclamation plants, Fannin in the U.S. (26) and Telsch and Katznelson (27) in Israel. Fannin attempted, unsuccessfully, to capture animal viruses from both trickling filter and waste activated sludge plants in Michigan. Telsch and Katznelson report capture of confirmed echovirus 7 in four out of twelve samples collected over a two week period. The samples were collected 40m downwind of a spray irrigation sprinkler which used secondary effluent. The Israeli workers effectively sampled more air than did Fannin by about a factor of 3.5. Fannin conceded in his discussion that the quantity of air which he sampled may not have been sufficient. No effort was made by the Israeli workers to quantitate the virus content of the air captured. Crude calculations, however, can be made using their data. These calculations give a minimum estimate of 3.4×10^{-2} virus mpn/m^3. This figure is some 2,000 times higher than the minimum estimate of animal virus in waste treatment plant aerosols made by Fannin (1976). These startling differences might be explainable on three counts: 1) Fannin (26) sampled aerosols from WRP having a much lower aerosolization efficiency than the spray irrigation apparatus utilized by Telsch and Katznelson (27). 2) Sewage in Israel is much more concentrated (10-20x) than sewage in the U.S. thus concentrations in the effluent could also be expected to be higher. 3) The effluent source is a large university hospital. Comparisons are difficult to make, however, since the Israeli

workers made no estimates of viral concentrations in their irrigation spray source.

In the present study the data are equivocal with respect to virus concentrations downwind of the sludge spray source (see Table 14). The animal virus concentrations appear to decrease with distance downwind of the sludge spray source. However, one upwind control sample was also positive for animal virus, at a concentration equivalent to that found at the intermediate downwind distance. To further confound the issue no animal virus was demonstrable in any of the sludge source samples (see Table 14). Furthermore, no animal virus was demonstrable in any sludge or soil specimens taken throughout the entire 15 months of the present study (see Tables 11, 13, and 14). Downwind air samples taken at the same time and at similar distances downwind were negative for animal virus (see Table 14). These similar samples processed more air than did the positive samples in two out of three instances (see Table 14).

The bacteriological data in Table 14 are also inconsistent, in that, at times air concentrations at the greatest downwind distances are greater than those at intermediate distances. Such inconsistency may be more apparent than real as all of the samples at the several distances on a given day may not have been taken simultaneously nor for the same length of time.

Sorber et al (28) compared total bacterial count from LEAP high volume samplers with the total bacterial count from Andersen six-stage impactors. The estimates of aerosol strength were essentially similar for both types of sampler. The data in the present study (Table 14) indicates that the LVAS sampler and the Andersen six-stage impactor may, in fact, be sampling different populations of particles.

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APPENDIX A

This section consists of statistical analyses pertaining to certain comparisons that reflect the effect of sludge application to land on the bacterial populations. The results are presented in separate subsections each of which is arranged to address itself to a particular aspect of the problem. The data corresponding to the levels of viral populations are not amenable to similar statistical analysis as the observed values are not different from zero. In what follows, we use the terms control fields for those fields that did not receive any sludge and the treated fields for those fields that received sludge.

The data presented in Table A1 pertaining to R-10 were obtained from the log books of MSDGC while the data pertaining to R3 and B-13-1 were obtained from Tables 3 and 7 of the text.

Effect of Runoff from Treated Fields on the Reservoirs

Reservoir R-3 receives runoff from sludge treated fields. Reservoir R-10 is a control field. If the bacterial populations present in the fields are transported by runoff, then the levels of these populations in the reservoirs that receive runoff from sludge treated fields will increase significantly when compared to those reservoirs that receive runoff from the control fields. To measure such an effect we employ the Wilcoxon rank sum statistic to test the hypothesis:

H: There is no difference in the levels of bacteria between R-3 and R-10.

against the alternative hypothesis

H_1: The levels of bacteria in R-3 are higher than those in R-10.

If n and m are the number of observations on R-3 and R-10 respectively, then using the large sample approximation we obtain the statistic

$$Z = \frac{W - E_H\ (W)}{SD_H\ (W)}$$

TABLE A1. LEVELS OF BACTERIA IN RESERVOIRS R10[1], R3, & B-13-7 DURING AUGUST 1975 TO SEPTEMBER 1976

Total Coliform*			Fecal Coliform*			Fecal Streptococcus*		
R 10	R 3	B-13-1	R 10	R 3	B-13-1	R 10	R 3	B-13-1
1.0	20.0	400.0	0.2	4.0	5.0	0.10	1.0	35.0
0.448	60.0	3700.0	0.1	2.0	1.0	0.344	70.0	14.0
1.28	2300.0	154.0	0.9	100.0	10.2	0.126	1.0	387.8
0.08	240.0	70.0		1.0	2.0	0.48	2.0	57.0
1.6	1.2			1.0		0.04	1.0	
	20.0			1.0			1.0	
	5.0			1.0			3.0	
	5.8			1.6			35.0	
	8.4			1.6			6.6	

	A	B	C	A	B	C	A	B	C
N	9	4		9	4		10	4	
M	5	9		3	9		5	10	
W	88.0	41.0	94	72.0	34.5	61.0	101.0	41.5	105.0
E_h (W)	67.5	28.0	50.5	58.5	28.0	64.0	80.0	30.0	55.0
$S.D_h$ (W)	7.5	6.4807	12.0381	5.4083	6.4807	9.8615	8.1649	7.071	12.9099
Z	2.733	2.006	3.6135	2.496	1.003	-0.304	2.572	1.626	3.973
P W_t W	0.002	0.025		0.005	0.207		0.004	0.071	

* Entries are expressed in terms of colony forming units/100 ml.

1 Data pertaining to R10 are obtained from MSDGC log books.

TABLE A2. LEVELS OF BACTERIA AT STATIONS S1 AND S2 DURING THE PERIOD AUGUST 1975 TO SEPTEMBER 1976

Total Coliform*		Fecal Coliform*		Fecal Streptococcus*	
S1	S2	S1	S2	S1	S2
2000.0	150.0	120.0	17.0	23.0	3.2
600.0	430.0	90.0	28.0	1.4	2.3
75.0	6.0	4.0	0.1	2.5	4.5
3100.0	1.0	390.0	1.0	5.0	0.1
1500.0	2300.0	6.0	23.0	17.0	1.8
190.0	1800.0	5.1	2.2	2.1	0.5
4200.0	100.0	130.0	230.0	86.0	70.0
100.0	90.0	22.0	19.0	10.0	6.0
190.0	6.0	5.6	3.6	1.8	1.0
15.0	14.0	3.9	6.0	2.4	2.4
120.0	49.0	15.0	6.0	1.1	1.8
170.0	65.0	7.5	5.2	9.1	7.5
510.0		15.0		2.1	4.0
N	12	12		13	
M	13	13		13	
W	116.5	137.0		156.0	
$E_h(W)$	156.0	156.0		175.5	
$S.D_h(W)$	18.3847	18.3847		19.5	
Z	-2.1485	-1.0334		-1.0	

* Entries are expressed in terms of colony forming units/liter.

where:

W = sum of the ranks associated with R-3

$$E_H\ (W) = m\ (m+n+1)/2$$

$$SD_H\ (W) = mn\ (m+n+1)/12$$

which is a standard normal variable. Also, for small values of n and m, the small sample exact probabilities of the statistic W are available. Using such tables we can evaluate $P[W \geq W_O]$ where W_O is any fixed value. Table A1 contains under the captions A and B, the statistics resulting from comparisons of R-3 and R-10; and R-3 and B-13-1, respectively. It can be seen from Column A that there exist significant increases in the levels of bacteria between R-3 and R-10 with respect to each of the variables - total coliform, fecal coliform and fecal streptococcus. Thus the runoff from treated field increases the bacterial levels in the reservoirs more than that from control fields. The small sample evaluations also confirm the same hypothesis in rending small probabilities (see $P\ (W_T \geq W)$ in column A).

It is conceivable that the proximity of a reservoir receiving runoff from the treated field might affect the bacterial populations in that as the distance increases the density of the bacterial population in the runoff decreases. Basin B-13-1 receives runoff directly from the treated fields and is located nearest to a set of treated fields while R-3 is located farther away. Informally we imply that the quality of runoff reaching R-3 is a diluted version of what is received by B-13-1 as far as the bacterial populations are concerned. A comparison of the levels of bacterial populations of R-3 and B-13-1 would provide evidence as to the tenability of this hypothesis. Column B of Table A1 gives the values of the statistics that test the hypothesis

H: There is no difference between the levels of bacterial populations of R-3 and B-13-1.

against the alternative hypothesis

H_1: The bacterial density in B-13-1 is higher than that of R-3.

The Wilcoxon rank sum statistic is computed as in the previous case. The results shows that there is no difference between B-13-1 and R-3 as far as fecal coliform and fecal streptococcus are concerned; however the total coliform levels in B-13-1 are higher than those in R-3.

Finally, it is of interest to find if there is any ordering of the bacterial levels among R-10, R-3, and B-13-1. In particular, we wish to test the hypothesis

H: There is no difference among the levels of bacteria of R-10, R-3, and B-13-1.

against the alternative hypothesis

H_1: The levels of bacteria are in increasing order of magnitude relative to R-10, R-3, and B-13-1.

The above hypothesis can be tested by the Jonckheere statistic:

$$W = \sum_{i<j}^{3} U_{ij}$$

where U_{ij} is the number of times the values in the i^{th} sample are preceded by the values in the j^{th} sample. For the present problem we employ the large sample approximation and obtain the standard normal variable

$$Z = \frac{W - E_H\ (W)}{SD_H\ (W)}$$

where $E_H\ (W)$ and $SD_H\ (W)$ are computed in the following way. If n_1, n_2, and n_3 demote the number of observations from the reservoirs R-10, R-3, and B-13-1, and $N(=n_1+n_2+n_3)$ denotes the total number of observation then

$$E_H\ (W) = (N^2 - \sum_{j=1}^{3} n_j^2)/4$$

$$SD_H\ (W) = ([N^2(2N+3) - \sum_{j=1}^{3} n_j^2\ (2n_j+3)]/72)^{1/2}$$

Column C in Table A1 gives the values of Z. With respect to the variables total coliform and fecal streptococcus the alternative hypothesis H_1 holds, while with respect to fecal coliform it seems that there is no evidence to reject the null hypothesis.

Effect of Sludge Application on the Stream

Sampling stations S-1 and S-2 were chosen so as to monitor the quality of water in a stream at the point of entry and exit of the property of the district. The differences, if they exist,

between the stations S-1 and S-2 with respect to any given variable (parameter) should reflect the effects of sludge application to the fields of MSDGC, with subsequent runoff to R-3 and to the stream.

Table A2 summarizes the data collected at stations S-1 and S-2. The Wilcoxon rank sum test is applied to test the hypothesis

H: There is no difference between S-1 and S-2 with respect to the levels of bacteria

against the alternative

H_1: The levels of bacteria are higher at S-2 when compared to S-1

The results show that the levels of total coliform are significantly lower at S-2 than at S-1 while there are no differences in the levels of fecal coliform and fecal streptococcus between the stations. This indicates that sludge application to the fields of MSDGC is not deteriorating the stream as far as these variables are concerned.

APPENDIX B

This section consists of the details pertaining to the statistical analysis of the data presented in Table 14 of the text. The data on coliphage and total bacterial counts were analyzed to obtain only functional relationships that might exist between the observed count and the distance of the sampler from the spray, and environmental factors such as wind velocity, temperature and relative humidity. The data on animal viruses were analyzed to test the effect of upwind conditions on downwind conditions. Each of these analyses is presented below:

A. Analysis of Animal Virus Data

Animal virus data were obtained by analyzing the samples collected employing the Litton sampler. These were placed at various distances upwind and downwind of the sludge sprayer. Upwind measurements are expected to reflect the conditions prior to spraying while the downwind measurements are expected to shed light on the rate at which the levels of animal virus decrease with increasing distance of the sampler from the source of spray.

For the purposes of this analysis we will call the event of detection of the presence of animal virus in a sample a success and the non-detection or absence of animal virus in a sample a failure. First, we will examine the data to test whether or not upwind conditions (conditions prior to spraying) affect the conditions downwind. If the upwind condition does not influence the downwind condition, then the probability of detecting the presence of animal virus downwind of the sprayer whether or not the virus is detected upwind remains the same. To express this in probabilistic terms, let A and B denote the event of obtaining a success downwind and upwind respectively; while $\bar{A}$ and $\bar{B}$ denote the events of obtaining a failure downwind and upwind respectively. Let P (A/B) denote the conditional probability of obtaining a success downwind given that a success has also been realized upwind; and a corresponding interpretation holds for P ($A/\bar{B}$). If the downwind condition is independent of the upwind condition then:

(1) $$P\ (A/B) = P\ (A/\bar{B})$$

To test whether the data can support the hypothesis in equation (1), we must make the following assumptions:

(a) For each downwind observation there exists a corresponding upwind observation. For this study, on any given day, several observations were made downwind while only one observation was made upwind. This assumption implies that repetitive upwind measurements would yield the same result and thus one can pair corresponding upwind, downwind observations.

(b) The observations made at the various distances downwind of the spray are indistinguishable in that we ignore the effect of distance on the observation. This implies that we can consider the downwind observations as a random sample.

Under these two assumptions we can derive the table below from the data presented in Table 14 of the text:

TABLE B1
OCCURRENCE OF ANIMAL VIRUS
UPWIND VERSUS DOWNWIND

Upwind	Downwind		
	Present (A)	Absent ($\bar{A}$)	Total
Present (B)	[X] 1	[X'] 0	[m] 1
Absent ($\bar{B}$)	[Y] 1	[Y'] 10	[n] 11
Total	[T] 2	[T'] 10	[N] 12

The letters within the brackets in Table B1 denote the general values for the entries. We reject the hypothesis presented in equation 1 if the conditional probability of X=x given X + Y = T is small. That is, if P[X=x/X+Y=t] is small. From Table B1 above, we found that P[X=1/X+Y=2] = 0.1666. Given that the marginal totals are fixed, the only other possible outcome of this experiment would have been one in which X = 0, X' = 1, Y = 2, and Y' = 9. For this outcome we find that P[X=0/X+Y=2] = 0.8333. Of these two likely events, the present experiment yield an outcome that is less probable which throws doubt on the

validity of the hypothesis of Equation (1). We are thus inclined to believe that the upwind conditions affect the downwind conditions, which implies that the spray is not the only source that might contribute animal viruses to the air currents.

It would have been of considerable interest for this experiment to study the effect of distance from the spray source on recovery of animal virus. Although such an analysis can be pursued using the available data, the validity of the results would be suspect in the light of the upwind versus downwind results which indicate that the sprayer may not be the only source which contributes animal virus.

The data for coliphage and total bacterial count were analyzed by regression techniques. The "goodness of fit" of a model, as far as these analyses are concerned, is judged by the square of the multiple correlation coefficient R. The various functions of the independent variables were selected to enter the model if R^2 were "significantly" improved; otherwise, the independent variable was dropped from the model in order to maintain a certain level of parsimony of the model. The models thus presented are the best in this sense and they are empirical models because of the regression technique.

B. Analysis of Coliphage and Total Bacterial Count Data

The aerosol samples collected on September 1, 1976 (presented in Table 14) were analyzed for coliphages by a method different from the one employed for previously collected samples. These five samples were collected at various distances from the spray source. Since the same sludge was sprayed, we can study the effect of distance of sampler from the spray source on the levels of coliphages captured in the sampler.

Let Y denote the level of coliphages captured in aerosol samples collected at a distance X from the spray source. Then based on above mentioned five observations, we obtain that $\log_e Y$ is linearly related to X through the equation.

(1) $$\log_e Y = 10.2178 - 0.0225\ X$$

This regression equation explains 95.6 percent (percent R^2) of the variation in $\log_e Y$. Thus, one can infer that if the sludge source remains the same, the levels of coliphages captured at a distance X from the sprayer exponentially decrease with X. The data collected on the other days are not amenable to such analysis because the distances at which the sampler was positioned were not selected in any schematic way.

However, it is desirable to know about the dispersion of the coliphages and total bacteria irrespective of the levels of

these parameters in sludge sources; that is, if the levels in sludge source are fixed, it is desirable to study the way in which the other environmental factors, such as wind velocity, distance of the sampler from the spray source, temperature and relative humidity, influence the levels observed in aerosols.

Let X_1 denote the level of either coliphages or the total bacteria in the sludge source. Let Y denote the level of corresponding parameter in the aerosol samples collected at a distance X_2 from the sprayer source. From the analyses of the data presented in Table 14, the regression model of the type

$$Y^p = B_0 + B_1 X_1^q + B_2 X_1^r + e \tag{2}$$

seems to explain the variation of Y adequately for either set of data (coliphages or total bacterial count). Here e's are normal random variables with mean zero and variance σ^2.

The model in (2) is suggested to an extent by the model obtained through the analyses of coliphage data and presented in equation (1) above, which says that a transformation of the form Y^p should be used and that the value of p should be small. The validity of the model presented in equation (2) is examined by using the data presented in Table 14. In analyzing the data the values of p, q, and r are selected by trial and error such that the value of R^2 is improved. From the data the regression coefficients B_0, B_1, and B_2 have been estimated. The data pertaining to coliphages, total bacterial count obtained by Litton sampler and that obtained by Andersen six-stage sampler are analyzed separately and the results are given below. The number of observations employed for the regression analyses is denoted by n.

Coliphage data:

$n = 22$

$p = 0.075$ $\quad q = 1.25$ $\quad r = 0.5$

$B_0 = 0.2326$ $\quad B_1 = 1.4024 \times 10^{-6}$ $\quad B_2 = 0.0147$

$R^2 = 0.7554$

Total bacterial count data:

Litton Sampler:

$n = 12$

$p = 0.075$ $\quad q = 0.75$ $\quad r = 0.5$

$B_0 = 1.8724$ $B_1 = 4.37 \times 10^{-8}$ $B_2 = 0.0187$

$R^2 = 0.6911$

Andersen six-stage sampler:

$n = 10$

$p = 0.075$ $q = 0.75$ $r = 0.5$

$B_0 = 1.8892$ $B_1 = 3.03 \times 10^{-8}$ $B_2 = 0.0197$

$R^2 = 0.4216$

From these results the model presented in equation (2) seems to explain adequately the levels of organisms in aerosols. Also, it appears that the results obtained by the Litton sampler and those obtained by the Andersen sampler differ significantly. Ideally, the parameters p, q, and r need to be estimated from the data, however, the number of samples collected are not sufficient enough to estimate as many as six parameters.

Further, attempts have been made to find if the other environmental factors such as wind velocity, temperature and relative humidity influence the levels found in aerosols. It appears, from the data, these factors have little effect on Y in that the value of R^2 is not improved to any significant extent. From this finding it can be concluded that during the sampling period these three factors remained relatively (with respect to the variation in the aerosol levels) stationary; and the observed variations within each of these factors can be considered insignificant enough to induce a noticeable effect in the aerosol levels.

The levels of total bacterial count in the sludge source varies from 2.2×10^7 cfu/l to 1.2×10^{10} cfu/l. If this variation can be considered not significant when compared to the variability of the method of estimation, then we can assume that the levels of total bacterial count remains stationary during the period of sampling. Under this assumption, it was found that temperature influences the levels of total bacterial count observed in the levels of aerosols captured downwind. Such a result indicates that it is important to have the sludge source in the model, as an independent variable.

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